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TRANSPLANTATIO JAPONICA

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First International Workshop On

FK-506
A POTENTIAL BREAKTHROUGH IN IMMUNOSUPPRESSION

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A Highly Sensitive Method to Assay FK-506 Levels in Plasma

K. Tamura, M. Kobayashi, K. Hashimoto, K. Kojima, K. Nagase, K. Iwasaki, T. Kaizu, H. Tanaka, and M. Niwa

FK-506, A MACROLIDE antibiotic obtained from *Streptomyces tsukubaensis*, strain No. 9993, was isolated from the soil of Tsukuba, northern Japan.[1] Its structure has been determined chemically[2,3] and by x-ray crystallography.[4] FK-506 has been shown to have strong immunosuppressive activity against a mixed lymphocyte reaction (MLR)[1] and promises to be useful in organ transplantation.[5] Its mechanism is considered to be a suppression of both interleukin 2 (IL-2) and IL-2 receptor expression on T cells.[1,6] Its activity has been reported to be 100 times more potent than that of cyclosporine A (CsA). As such, a sensitive assay method for a more potent immunosuppressant of FK-506 is crucial for pharmacokinetic studies and for setting effective administration dosages.

Here, we report a simple, safe, and sensitive enzyme immunoassay (EIA) for FK-506. The pharmacokinetics were also monitored after low-dose administration of FK-506 in dogs.

MATERIALS AND METHODS

Preparation of FK-506 Hemisuccinate

FK-506 hemisuccinate (90 mg) was prepared from 248 mg FK-506 and 145 mg succinic anhydride with 4-dimethylaminopyridine in pyridine.

Preparation of Activated Ester of FK-506 Hemisuccinate

The activated ester (74.1 mg) was prepared by reacting 90 mg of FK-506 hemisuccinate and 12.7 mg of *N*-hydroxysuccinimide with dicyclohexylcarbodiimide in ethyl acetate.

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Preparation of Horseradish Peroxidase–FK-506 Conjugate

An activated ester solution of FK-506 (0.48 mg) in dioxane (10 μL) was mixed with a solution of horseradish peroxidase (POD; type IV, Sigma Chemical Co, St Louis, MO; 10 mg) in 0.18 mL of dioxane and 0.18 mL of 0.5% $NaHCO_3$ solution and stirred at 4°C for 2.5 hours. To this reaction mixture, 1.77 mL of 50 mmol/L PB (pH 7.0) containing 0.1% (wt/vol) gelatine was added, and the solution was dialyzed against 50 mmol/L PB (pH 7.0) completely. This POD–FK-506 solution was diluted 2×10^5–fold with 1% (wt/vol) BSA in phosphate buffered saline (PBS) (1% BSA-PBS) and used in the assay.

Preparation of Polyclonal Antibody to FK-506

The BSA–FK-506 conjugate (1.6 mg as BSA) in PBS solution (2.5 mL) was used to immunize a New Zealand white rabbit with Freund's complete adjuvant (FCA) (2.5 mL) and was followed by a couple of boosters containing a suspension of BSA–FK-506 conjugate (1.6 mg as BSA) in PBS (2.5 mL) and Freund's incomplete adjuvant (FIA) (2.5 mL). Serum with high antibody titers and with suppressive activity to FK-506 MLR inhibition was collected, and a crude IgG fraction was precipitated at 33% $(NH_4)_2SO_4$. IgG was purified on DE-52 columns in 20 mmol/L PB (pH 8.0). Fractions that passed through the column were collected and used for EIA as purified polyclonal antibody (PcAb) against FK-506.

Preparation of Monoclonal Antibody to FK-506

A PBS solution (0.2 mL) of the BSA–FK-506 conjugate (50 μg as BSA) was used to immunize BALB/c mice

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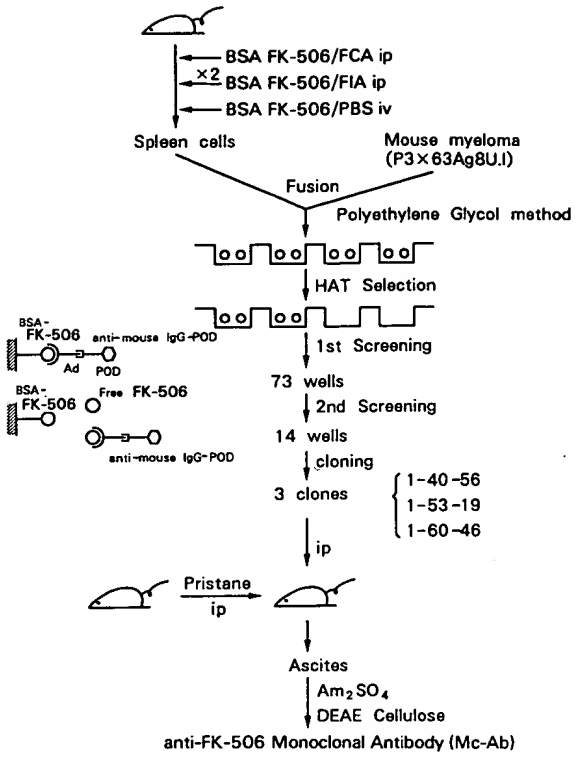

Fig 1. Preparation of mouse MoAb to FK-506.

with 0.2 mL of FCA (Fig 1). The mice were boosted a couple of times with intraperitoneal injections of BSA–FK-506 (0.2 mL; 50 μg as BSA) and FIA (0.2 mL). Three days after the last boosting with an intravenous injection of BSA–FK-506 (0.2 mL; 200 μg as BSA), the spleen was excised and used for cell fusion. The spleen cells were fused with mouse myeloma cells (P3X63Ag8U.1) at a 5:1 ratio with 45% polyethylene glycol. After hypoxanthine-aminopterin-thymidine (HAT) selection, hybridoma cells were screened for specific IgG against FK-506 and not against BSA. Clones whose supernatant showed positive reactions in their affinity to BSA–FK-506 but became negative in the presence of excess free FK-506 were selected. After cloning by the limiting dilution method, three clones were expanded in the peritoneal cavity of pristane-primed BALB/c mice, and IgG was purified by $(NH_4)_2SO_4$ precipitation and DE-52 chromatography. Each purified IgG was characterized by the Ouchterlony method, and their detectability for FK-506 was tested in one-step EIA.

Two-Step EIA Procedure

First, antimouse IgG Ab was adsorbed onto a 96-well microtiter plate (Sumitomo Bakelite MS-3596F, Tokyo) by incubating the plate overnight at 4°C with 200 μL of antimouse IgG Ab in PBS (3 μg/mL) per well. The antibody solution was aspirated off, 300 μL of 1% BSA-PBS solution was added, and the plate was incubated for 30 minutes at 37°C to cover all nonspecific binding sites. The solution was substituted with 100 μL of the 2 × 10^5-fold–diluted solution of POD–FK-506 diluted with 1% BSA-PBS. To this solution in the well was added 100 μL of FK-506 solution (FK-506 standard solution appropriately diluted with 10% normal plasma in 1% BSA-PBS or a tenfold-diluted plasma sample in 1% BSA-PBS from an animal treated with FK-506). Then, 50 μL of monoclonal antibody (MoAb) solution (10 ng/mL) in 1% BSA-PBS was added to each well, and the plate was incubated at 4°C overnight. The solution was aspirated off, and the plate was washed with 0.05% Tween 20–PBS and washed again with PBS. The enzyme substrate solution was added, and the plate was left at room temperature for 30 to 60 minutes until a good optical density of 492 nm (OD_{492}) was attained. The colorimetric reaction was stopped by the addition of 50 μL of a 4 N H_2SO_4 aqueous solution. The OD_{492} was measured and plotted on a semilogarithmic graph. The concentration of FK-506 is on the abscissa of the logarithmic scale, and OD_{492} is on the ordinate, of the normal scale.

Enzyme substrate solution was prepared by dissolving 100 mg of *o*-phenylenediamine hydrochloride and 50 μL of 30% H_2O_2 in 100 mL of phosphate-citrate buffer (pH 5.4). The phosphate-citrate buffer (pH 5.4) used in this experiment was prepared by adjusting the pH of a 0.1 mol/L Na_2HPO_4 aqueous solution with a 0.1 mol/L citric acid aqueous solution to pH 5.4.

One-Step EIA Procedure

The PcAb or MoAb concentration used in one-step EIA was 20 μg/mL in PBS. Competitive reaction between free FK-506 and POD–FK-506 was started by the addition of these two components. The concentration of POD–FK-506 and the rest of the procedure were the same as those in two-step EIA (Fig 2).

RESULTS

Preparation of PcAb to FK-506

After the fourth immunization, the titer (the dilution fold that causes 50% decrease of antibody binding to BSA-FK-506) was 10^4. From 340 mL of antiserum, 5,646 mg of PcAb to FK-506 was obtained by purification as described in Materials and Methods.

Preparation of MoAb to FK-506

After HAT selection and two screenings, three hybridomas that produced MoAbs specific against FK-506 were cloned. Each of these monoclonal hybridomas, 1-40-56, 1-53-19, and 1-60-46, was expanded in the perito-

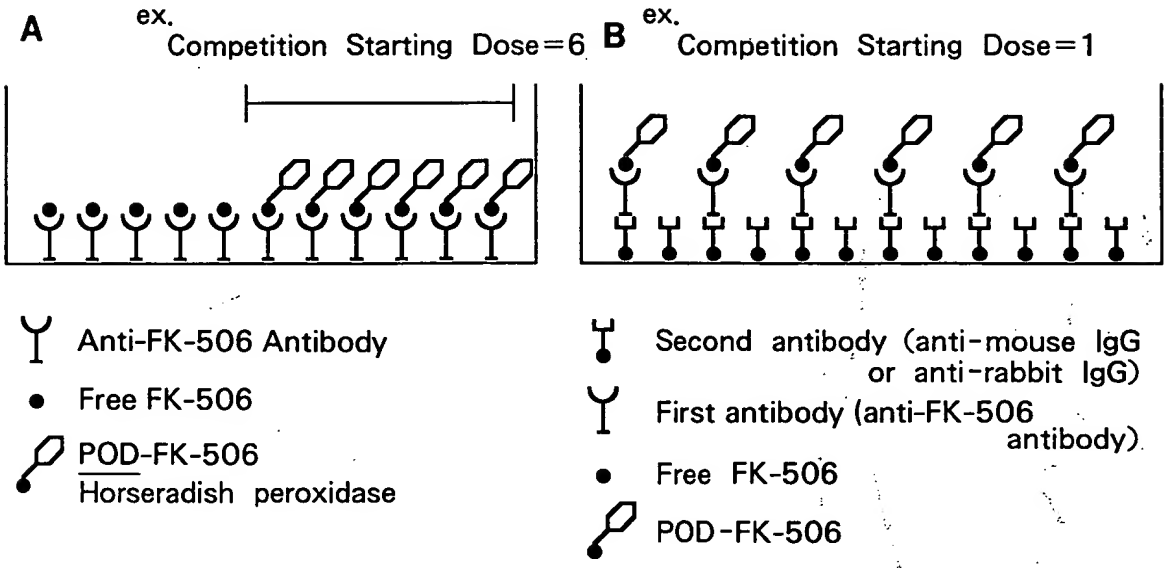

Fig 2. Illustration of (A) one-step EIA and (B) two-step EIA.

neal cavity of pristane-primed BALB/c mice and yielded respective purified anti–FK-506 MoAbs after conventional purification. The data of MoAb production and their characteristics are shown in Table 1.

Comparison of One-Step EIA With PcAb and MoAb

The results of one-step EIA with PcAbs and MoAbs are shown in Fig 3. PcAb gave the highest sensitivity, and the minimum detection level was 0.33 ng/mL in 10% plasma. Of the MoAbs, MoAb 1-60-46 gave the best sensitivity, which was 1 ng/mL in 10% plasma. The most striking difference between MoAb 1-60-46 and PcAb was that the standard curve was steeper with the MoAb, with a detection range from 1 ng/mL in 10% plasma to 100 ng/mL in 10% plasma, whereas that with PcAb was shallow and gave a wider detection range from 0.33 ng/mL in 10% plasma to 10^3 ng/mL in 10% plasma.

Table 1. MoAb Production and Their Subclass

MoAb	Ascites (mL)	Purified IgG (mg)	IgG Subclass
1-40-56	11	125.5	IgG1
1-53-19	6	18.7	IgG2b
1-60-46	40	175.0	IgG1

Precision of One-Step EIA Using PcAb

Intraassay and interassay variances were assessed from the data with appropriately diluted FK-506 in 10% plasma and are shown in Table 2. The coefficients of variance were 5.8% to 24.5% for intraassay and 4.0% to 11.5% for interassay.

Effect of Plasma Concentration on the Assay and Elimination of the Effect by Benzene Extraction

The EIA sensitivity was found to depend heavily on the plasma concentration (Fig 4). When FK-506 in 100% plasma was diluted tenfold, the minimum detectable level was almost the same as that in 1% BSA-PBS, but this means that the sensitivity was lowered by tenfold. This interference by plasma concentrations over 10% was eliminated by benzene extraction of the plasma sample (Fig 4); dichloromethane extraction could also eliminate the effect (data not shown).

More Highly Sensitive Assay System of Two-Step EIA

When the two-step EIA was applied to PcAb, the minimum detectable level came down to 1 pg/mL, a 300-fold improvement in

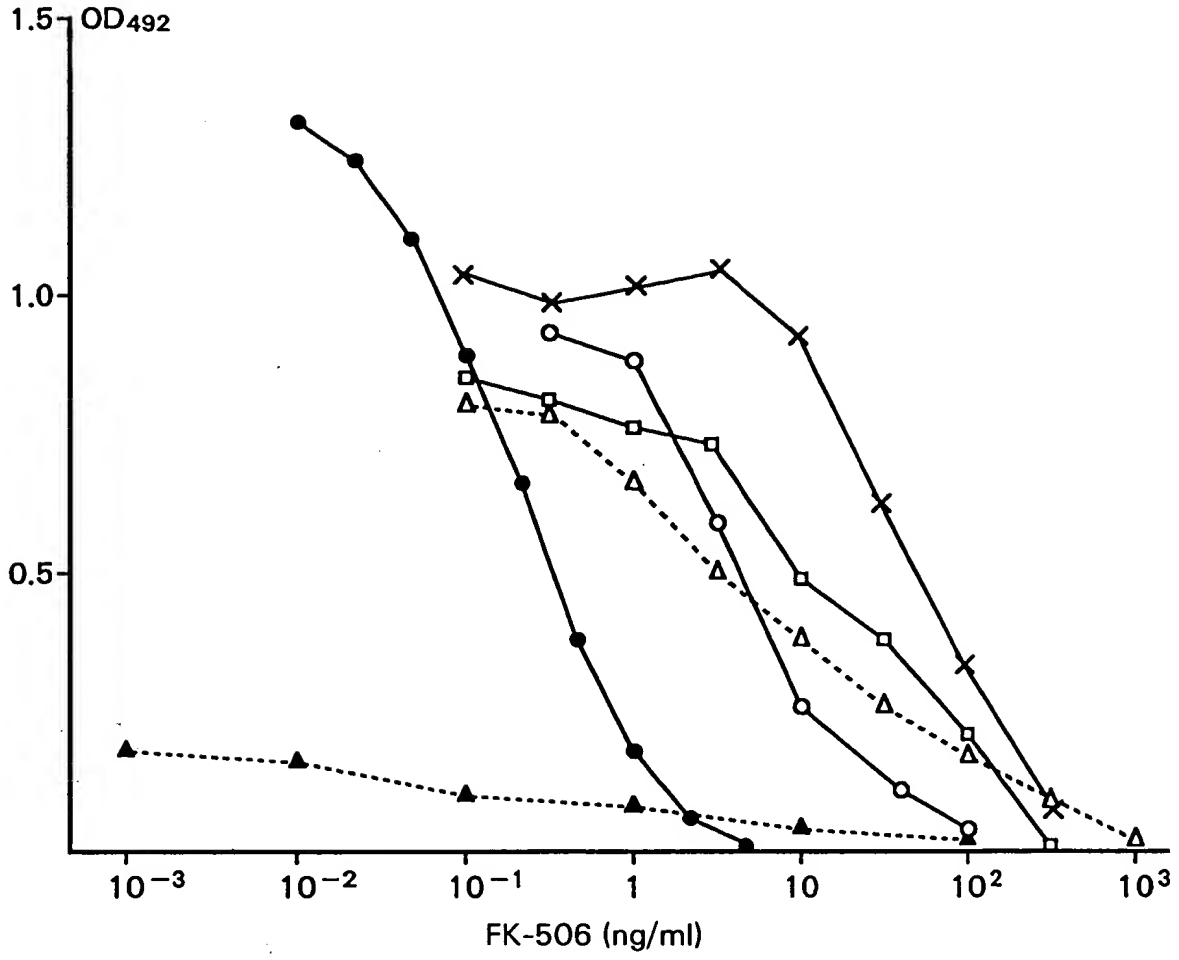

Fig 3. Standard curves of FK-506 by two-step EIA using PcAb and MoAb 1-60-46 and those by one-step EIA using PcAb and three MoAbs. △-----△, PcAb one-step EIA; X—X, MoAb 1-40-56 one-step EIA; □—□, MoAb 1-53-19 one-step EIA; ○—○, MoAb 1-60-46 one-step EIA; ▲-----▲, PcAb two-step EIA; ●—●, MoAb 1-60-46 two-step EIA.

the sensitivity. Next, MoAb 1-60-46 was examined for its applicability to this two-step EIA. As a second Ab, rabbit PcAb to mouse IgG prepared in our laboratory was used. In this assay with MoAb 1-60-46, a good minimum detectable level of 20 pg/mL in 10% plasma was obtained. Again, the calibration curve for standard FK-506 in 10% plasma was steeper than that with PcAb (Fig 3).

Precision of Two-Step EIA With MoAbs

The intraassay and interassay coefficients of variance were 6.0% to 11.7% and 4.9% to 23.1%, respectively (Table 3).

Table 2. Precision of PcAb One-Step EIA: Coefficients of Variance for FK-506 in 10% Dog Plasma

	Intraassay (n = 7)		Interassay (n = 4)	
Sample	Mean ± SD (ng/mL)	CV (%)	Mean ± SD (ng/mL)	CV (%)
A	0.388 ± 0.0517	13.4	0.389 ± 0.0448	11.5
B	2.670 ± 0.4619	17.3	2.630 ± 0.1578	6.0
C	23.9 ± 5.858	24.5	22.7 ± 0.9032	4.0
D	80.5 ± 4.650	5.8	81.4 ± 4.540	5.6

Abbreviation: CV, coefficient of variance.

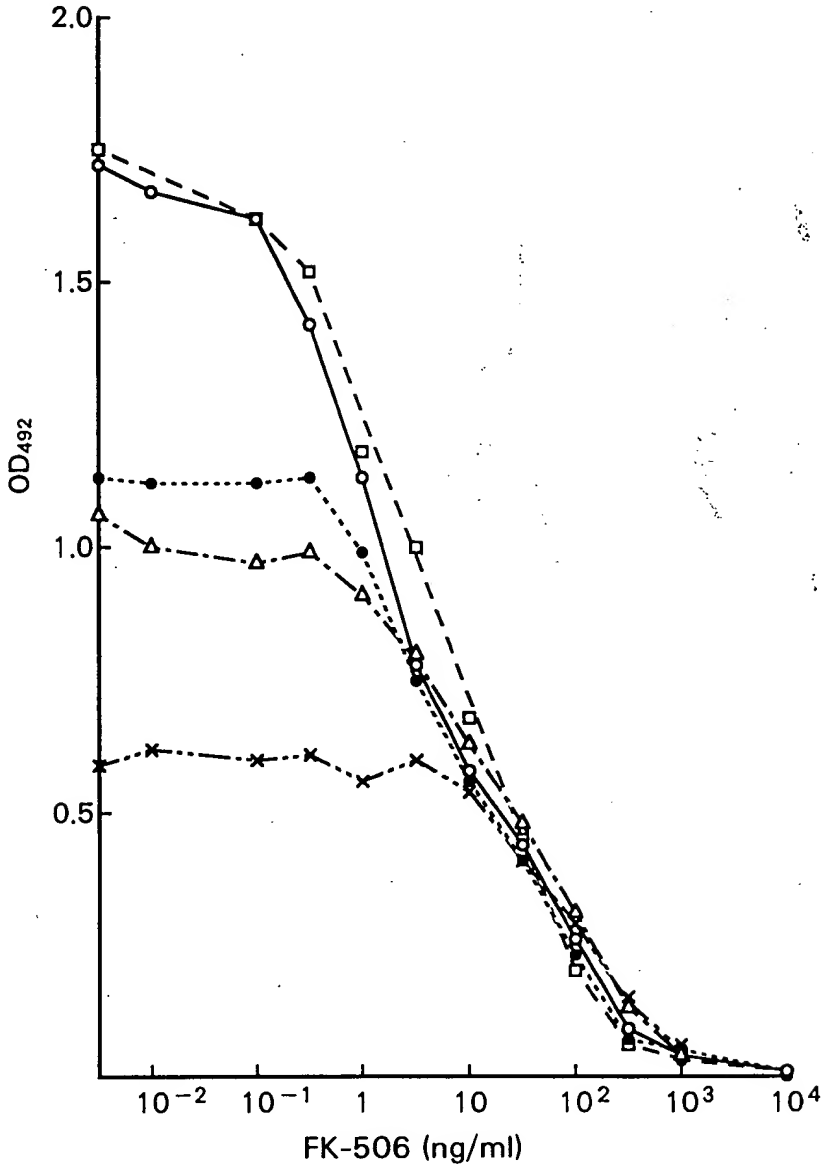

Fig 4. Influence of plasma concentration on the one-step EIA of FK-506 and elimination of the influence by benzene extraction. O—O, FK-506 in 1% BSA-PBS; ●----●, FK-506 in 10% dog plasma 1% BSA-PBS; △-—-△, FK-506 in 20% dog plasma 1% BSA-PBS; X—--—X, FK-506 in 50% dog plasma 1% BSA-PBS; □——□, benzene extraction from plasma.

Pharmacokinetics of FK-506 in Dogs After Oral Administration—Sensitive Two-Step EIA

The plasma concentration of FK-506 after 0.32 or 1 mg/kg administration of oral formulation (solid dispersion formulation [SDF][7]) was measured by two-step EIA after extraction pretreatment and was shown in Fig 5. In these cases, the plasma concentration of FK-506 could not be measured by one-step EIA. Oral administration gave a peak of FK-506

Table 3. Precision of MoAb Two-Step EIA: Coefficients of Variance for FK-506 in 10% Dog Plasma

	Intraassay (n = 6)		Interassay (n = 3)	
Sample	Mean ± SD (ng/mL)	CV (%)	Mean ± SD (ng/mL)	CV (%)
A	0.040 ± 0.0039	9.8	0.042 ± 0.0097	23.1
B	0.119 ± 0.0140	11.7	0.126 ± 0.0087	6.9
C	0.642 ± 0.0408	6.4	0.668 ± 0.0327	4.9
D	2.49 ± 0.1484	6.0	2.31 ± 0.3350	14.5

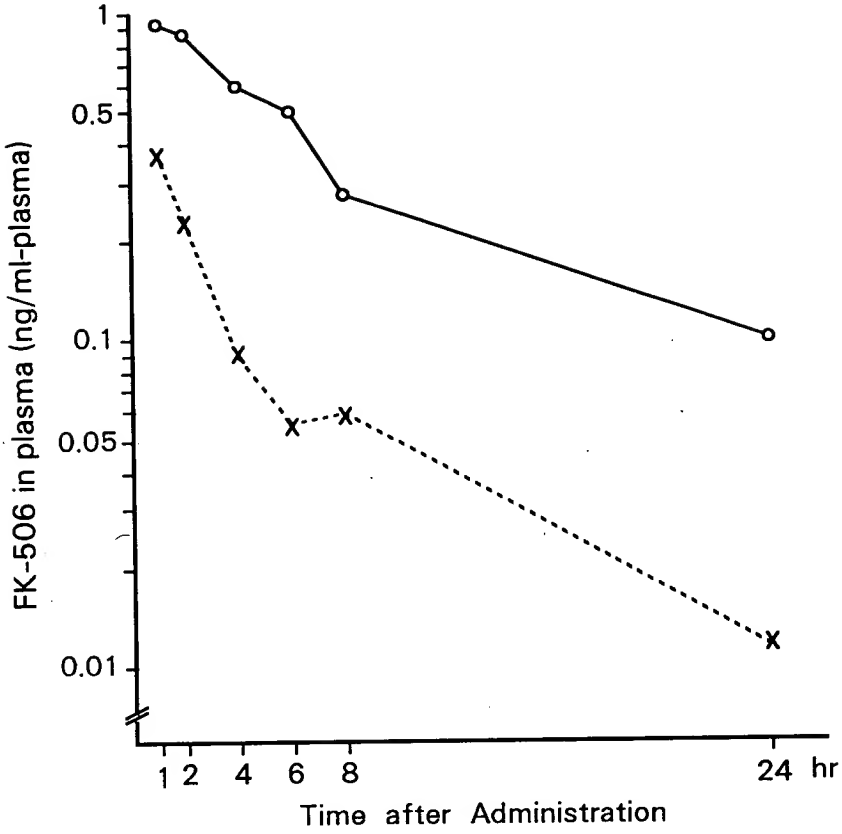

Fig 5. Pharmacokinetics of FK-506 following bolus oral administration of FK-506. X------X, 0.32 mg/kg; O—O, 1 mg/kg.

within one hour after administration of either dosage, and at 24 hours, the FK-506 concentration was 10 pg/mL at a dose of 0.32 mg/kg and 100 pg/mL at 1 mg/kg.

DISCUSSION

Rabbit PcAb and three mouse MoAbs against FK-506 were prepared by using BSA–FK-506 as a hapten-carrier antigen. By using these antibodies, two EIA systems were established: one-step EIA and two-step EIA (Fig 2).

Anti–FK-506 Ab is adsorbed onto the plate first in one-step EIA, and the second Ab of antimouse IgG or antirabbit IgG is adsorbed onto the plate first in two-step EIA. As a result, we have four combinations of Abs and assay systems: PcAb one-step EIA, PcAb two-step EIA, MoAb one-step EIA, and MoAb two-step EIA. Of three MoAbs, MoAb 1-60-46 gave the best sensitivity in one-step EIA (Fig 3) and was chosen for further study. But even with this MoAb, the sensitivity (1 ng/mL in 10% plasma) in one-step EIA was inferior to that of PcAb (0.33 ng/mL in 10% plasma). This PcAb one-step EIA method is rather simple and has satisfactory intraassay (5.8% to 24.5%) and interassay (4.0% to 11.5%) coefficients of variance when the wide detection range of 0.33 to 10^3 ng/mL in 10% plasma was taken into account, but it was still not sensitive enough to monitor the plasma FK-506 concentration at effective doses in dogs. When the mechanism of EIA is considered, higher sensitivity can be expected with lower concentrations of POD–FK-506. But if the number of anti–FK-506 Abs adsorbed on the plate is large compared with that of POD–FK-506, the free FK-506 might occupy the extra open anti–FK-506 Ab (Fig 2). In this sense, the smaller the number of the bound anti–FK-506 Ab (either PcAb or MoAb), the higher the sensitivity of the EIA. But it is difficult to reduce the number of bound anti–

FK-506 Ab on the plate accurately by only reducing the concentration ratio of the Ab by mixing with another protein such as BSA (data not shown). Thus, we tried to lower the number of anti–FK-506 Ab bound on the plate by using a second Ab to anti–FK-506 Ab. When a combination of antirabbit IgG Ab and anti–FK-506 PcAb (rabbit) was used, the minimum detectable level rose to 1 pg/mL in 10% plasma (Fig 3). When the combination of antimouse IgG Ab and anti–FK-506 MoAb was used, the minimum detectable level rose to 20 pg/mL in 10% plasma. As is evident from Fig 3, the maximum OD_{492} was as low as 0.2 for the former combination, whereas the maximum OD_{492} for the latter combination was the normal level of 1.5. Because it was considered that, when the maximum OD_{492} is low, slight errors in reading the OD_{492} might cause a larger error in FK-506 concentration, we concluded that a combination of anti–FK-506 MoAb and antimouse IgG Ab was the one to be adopted for two-step EIA. The minimum detectable level of 20 pg/mL is a 16-fold improvement over the 0.33 ng/mL by PcAb one-step EIA and a 50-fold improvement over the 1 ng/mL of MoAb one-step EIA (Table 4).

The new method of two-step EIA afforded a highly sensitive assay method. This technique is considered not to be limited only to FK-506 but can be applied to any enzyme immunoassay that requires very high sensitivity. By using this highly sensitive EIA, we measured the plasma concentration of FK-506 after 0.32 and 1 mg/kg oral administration (SDF). As shown in Fig 5, the FK-506 plasma concentration was clearly monitored after bolus administration. With 0.32 mg/kg orally (SDF), a peak plasma concentration (0.4 ng/mL) was attained one hour after administration, and a concentration of 0.09 to 0.06 ng/mL was maintained from four to eight hours, whereas with 1 mg/kg orally (SDF), the plasma peak level was 0.9 ng/mL one hour after administration, and concentrations of 0.6 to 0.3 ng/mL were maintained from four to eight hours.

Table 4. Summary of Minimum Detection Level

Assay	Antibody	Sample Pretreatment: Plasma Direct Method	Sample Pretreatment: Extraction Method
One-step EIA	MoAB	10 ng/mL	—
	PcAb	3.3 ng/mL	330 pg/mL
Two-step EIA	MoAb	200 pg/mL	20 pg/mL
	PcAb	10 pg/mL	1 pg/mL

Values were expressed in concentrations of dog plasma.

Here we have shown a highly sensitive new two-step EIA and that the pharmacokinetics of low-dose FK-506 can be monitored. The best dose form and route of FK-506 administration should be determined in accord with its pharmacokinetics.

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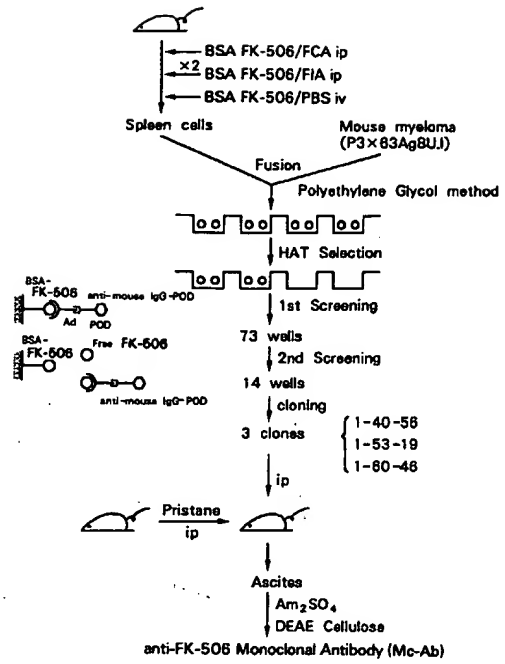

Fig 1. Preparation of mouse MoAb to FK-506.

with 0.2 mL of FCA (Fig 1). The mice were boosted a couple of times with intraperitoneal injections of BSA–FK-506 (0.2 mL; 50 μg as BSA) and FIA (0.2 mL). Three days after the last boosting with an intravenous injection of BSA–FK-506 (0.2 mL; 200 μg as BSA), the spleen was excised and used for cell fusion. The spleen cells were fused with mouse myeloma cells (P3X63Ag8U.1) at a 5:1 ratio with 45% polyethylene glycol. After hypoxanthine-aminopterin-thymidine (HAT) selection, hybridoma cells were screened for specific IgG against FK-506 and not against BSA. Clones whose supernatant showed positive reactions in their affinity to BSA–FK-506 but became negative in the presence of excess free FK-506 were selected. After cloning by the limiting dilution method, three clones were expanded in the peritoneal cavity of pristane-primed BALB/c mice, and IgG was purified by $(NH_4)_2SO_4$ precipitation and DE-52 chromatography. Each purified IgG was characterized by the Ouchterlony method, and their detectability for FK-506 was tested in one-step EIA.

Two-Step EIA Procedure

First, antimouse IgG Ab was adsorbed onto a 96-well microtiter plate (Sumitomo Bakelite MS-3596F, Tokyo) by incubating the plate overnight at 4°C with 200 μL of antimouse IgG Ab in PBS (3 μg/mL) per well. The antibody solution was aspirated off, 300 μL of 1% BSA-PBS solution was added, and the plate was incubated for 30 minutes at 37°C to cover all nonspecific binding sites. The solution was substituted with 100 μL of the 2 × 10^5-fold–diluted solution of POD–FK-506 diluted with 1% BSA-PBS. To this solution in the well was added 100 μL of FK-506 solution (FK-506 standard solution appropriately diluted with 10% normal plasma in 1% BSA-PBS or a tenfold-diluted plasma sample in 1% BSA-PBS from an animal treated with FK-506). Then, 50 μL of monoclonal antibody (MoAb) solution (10 ng/mL) in 1% BSA-PBS was added to each well, and the plate was incubated at 4°C overnight. The solution was aspirated off, and the plate was washed with 0.05% Tween 20–PBS and washed again with PBS. The enzyme substrate solution was added, and the plate was left at room temperature for 30 to 60 minutes until a good optical density of 492 nm (OD_{492}) was attained. The colorimetric reaction was stopped by the addition of 50 μL of a 4 N H_2SO_4 aqueous solution. The OD_{492} was measured and plotted on a semilogarithmic graph. The concentration of FK-506 is on the abscissa of the logarithmic scale, and OD_{492} is on the ordinate, of the normal scale.

Enzyme substrate solution was prepared by dissolving 100 mg of *o*-phenylenediamine hydrochloride and 50 μL of 30% H_2O_2 in 100 mL of phosphate-citrate buffer (pH 5.4). The phosphate-citrate buffer (pH 5.4) used in this experiment was prepared by adjusting the pH of a 0.1 mol/L Na_2HPO_4 aqueous solution with a 0.1 mol/L citric acid aqueous solution to pH 5.4.

One-Step EIA Procedure

The PcAb or MoAb concentration used in one-step EIA was 20 μg/mL in PBS. Competitive reaction between free FK-506 and POD–FK-506 was started by the addition of these two components. The concentration of POD–FK-506 and the rest of the procedure were the same as those in two-step EIA (Fig 2).

RESULTS

Preparation of PcAb to FK-506

After the fourth immunization, the titer (the dilution fold that causes 50% decrease of antibody binding to BSA-FK-506) was 10^4. From 340 mL of antiserum, 5,646 mg of PcAb to FK-506 was obtained by purification as described in Materials and Methods.

Preparation of MoAb to FK-506

After HAT selection and two screenings, three hybridomas that produced MoAbs specific against FK-506 were cloned. Each of these monoclonal hybridomas, 1-40-56, 1-53-19, and 1-60-46, was expanded in the perito-

ided, and the plate was incubated for
to cover all nonspecific binding sites.
ubstituted with 100 μL of the 2 ×
lution of POD–FK-506 diluted with
his solution in the well was added 100
ion (FK-506 standard solution appro-
1 10% normal plasma in 1% BSA-PBS
plasma sample in 1% BSA-PBS from
with FK-506). Then, 50 μL of mono-
MoAb) solution (10 ng/mL) in 1%
led to each well, and the plate was
vernight. The solution was aspirated
as washed with 0.05% Tween 20–PBS
with PBS. The enzyme substrate
d, and the plate was left at room
) to 60 minutes until a good optical
OD_{492}) was attained. The colorimetric
ed by the addition of 50 μL of a 4 N
lution. The OD_{492} was measured and
garithmic graph. The concentration
abscissa of the logarithmic scale, and
inate, of the normal scale.
te solution was prepared by dissolving
lenediamine hydrochloride and 50 μL
0 mL of phosphate-citrate buffer (pH
te-citrate buffer (pH 5.4) used in this
repared by adjusting the pH of a 0.1
queous solution with a 0.1 mol/L citric
ion to pH 5.4.

Procedure

MoAb concentration used in one-step
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RESULTS

PcAb to FK-506

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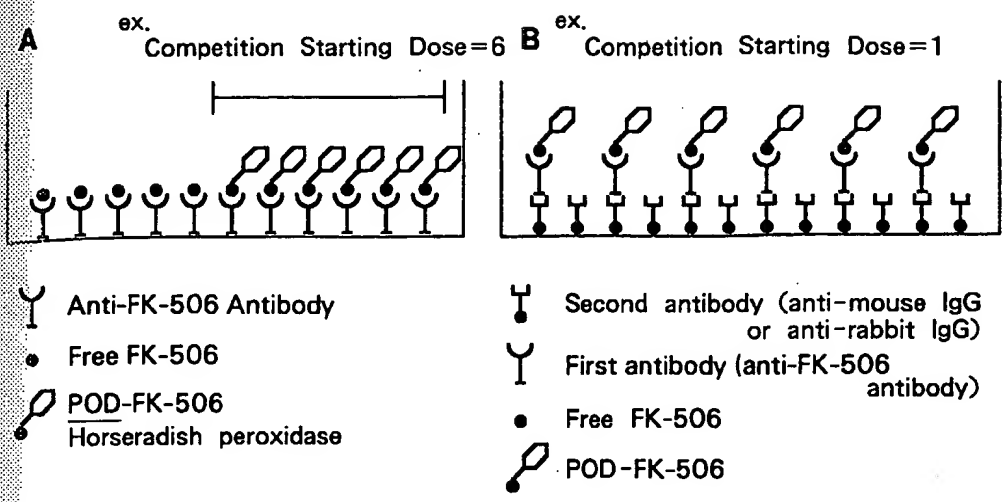


Fig 2. Illustration of (A) one-step EIA and (B) two-step EIA.

neal cavity of pristane-primed BALB/c mice and yielded respective purified anti–FK-506 MoAbs after conventional purification. The data of MoAb production and their characteristics are shown in Table 1.

Comparison of One-Step EIA With PcAb and MoAb

The results of one-step EIA with PcAbs and MoAbs are shown in Fig 3. PcAb gave the highest sensitivity, and the minimum detection level was 0.33 ng/mL in 10% plasma. Of the MoAbs, MoAb 1-60-46 gave the best sensitivity, which was 1 ng/mL in 10% plasma. The most striking difference between MoAb 1-60-46 and PcAb was that the standard curve was steeper with the MoAb, with a detection range from 1 ng/mL in 10% plasma to 100 ng/mL in 10% plasma, whereas that with PcAb was shallow and gave a wider detection range from 0.33 ng/mL in 10% plasma to 10^3 ng/mL in 10% plasma.

Table 1. MoAb Production and Their Subclass

MoAb	Ascites (mL)	Purified IgG (mg)	IgG Subclass
1-40-56	11	125.5	IgG1
1-53-19	6	18.7	IgG2b
1-60-46	40	175.0	IgG1

Precision of One-Step EIA Using PcAb

Intraassay and interassay variances were assessed from the data with appropriately diluted FK-506 in 10% plasma and are shown in Table 2. The coefficients of variance were 5.8% to 24.5% for intraassay and 4.0% to 11.5% for interassay.

Effect of Plasma Concentration on the Assay and Elimination of the Effect by Benzene Extraction

The EIA sensitivity was found to depend heavily on the plasma concentration (Fig 4). When FK-506 in 100% plasma was diluted tenfold, the minimum detectable level was almost the same as that in 1% BSA-PBS, but this means that the sensitivity was lowered by tenfold. This interference by plasma concentrations over 10% was eliminated by benzene extraction of the plasma sample (Fig 4); dichloromethane extraction could also eliminate the effect (data not shown).

More Highly Sensitive Assay System of Two-Step EIA

When the two-step EIA was applied to PcAb, the minimum detectable level came down to 1 pg/mL, a 300-fold improvement in

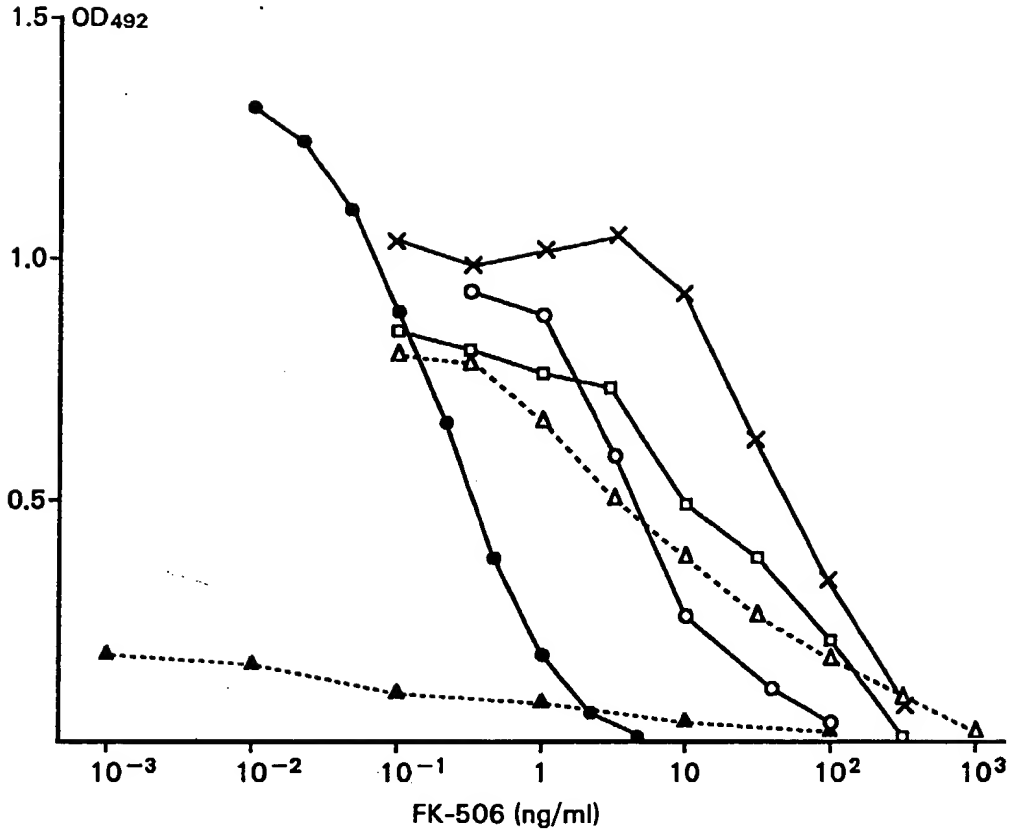


Fig 3. Standard curves of FK-506 by two-step EIA using PcAb and MoAb 1-60-46 and those by one-step EIA using PcAb and three MoAbs. △----△, PcAb one-step EIA; X—X, MoAb 1-40-56 one-step EIA; □—□, MoAb 1-53-19 one-step EIA; ○—○, MoAb 1-60-46 one-step EIA; ▲----▲, PcAb two-step EIA; ●—●, MoAb 1-60-46 two-step EIA.

the sensitivity. Next, MoAb 1-60-46 was examined for its applicability to this two-step EIA. As a second Ab, rabbit PcAb to mouse IgG prepared in our laboratory was used. In this assay with MoAb 1-60-46, a good minimum detectable level of 20 pg/mL in 10% plasma was obtained. Again, the calibration curve for standard FK-506 in 10% plasma was steeper than that with PcAb (Fig 3).

Precision of Two-Step EIA With MoAbs

The intraassay and interassay coefficients of variance were 6.0% to 11.7% and 4.9% to 23.1%, respectively (Table 3).

Table 2. Precision of PcAb One-Step EIA: Coefficients of Variance for FK-506 in 10% Dog Plasma

	Intraassay (n = 7)		Interassay (n = 4)	
Sample	Mean ± SD (ng/mL)	CV (%)	Mean ± SD (ng/mL)	CV (%)
A	0.388 ± 0.0517	13.4	0.389 ± 0.0448	11.5
B	2.670 ± 0.4619	17.3	2.630 ± 0.1578	6.0
C	23.9 ± 5.858	24.5	22.7 ± 0.9032	4.0
D	80.5 ± 4.650	5.8	81.4 ± 4.540	5.6

Abbreviation: CV, coefficient of variance.

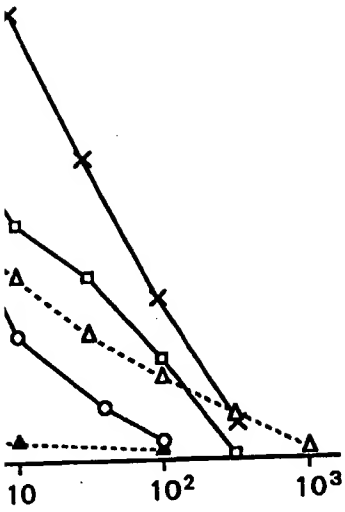
Fig 4. Influence of concentration on the EIA of FK-506 and elim of the influence by extraction. O—O, FK 1% BSA-PBS; ●—●, in 10% dog plasma 1% PBS; △----△, FK-506 dog plasma 1% BS X———X, FK-506 in 5 plasma 1% BSA □——□, benzene ext from plasma.

Pharmacokinetics After Oral Adminis Two-Step EIA

The plasma conc 0.32 or 1 mg/kg adr

Table 3. Precis

Sample
A
B
C
D



ıAb 1-60-46 and those by one-step EIA
ı 1-40-56 one-step EIA; □—□, MoAb
b two-step EIA; ●—●, MoAb 1-60-46

ıdard FK-506 in 10% plasma was
that with PcAb (Fig 3).

Two-Step EIA With MoAbs

ıssay and interassay coefficients
were 6.0% to 11.7% and 4.9% to
ctively (Table 3).

ı for FK-506 in 10% Dog Plasma

Interassay (n = 4)	
Mean ± SD (ng/mL)	CV (%)
).389 ± 0.0448	11.5
2.630 ± 0.1578	6.0
22.7 ± 0.9032	4.0
81.4 ± 4.540	5.6

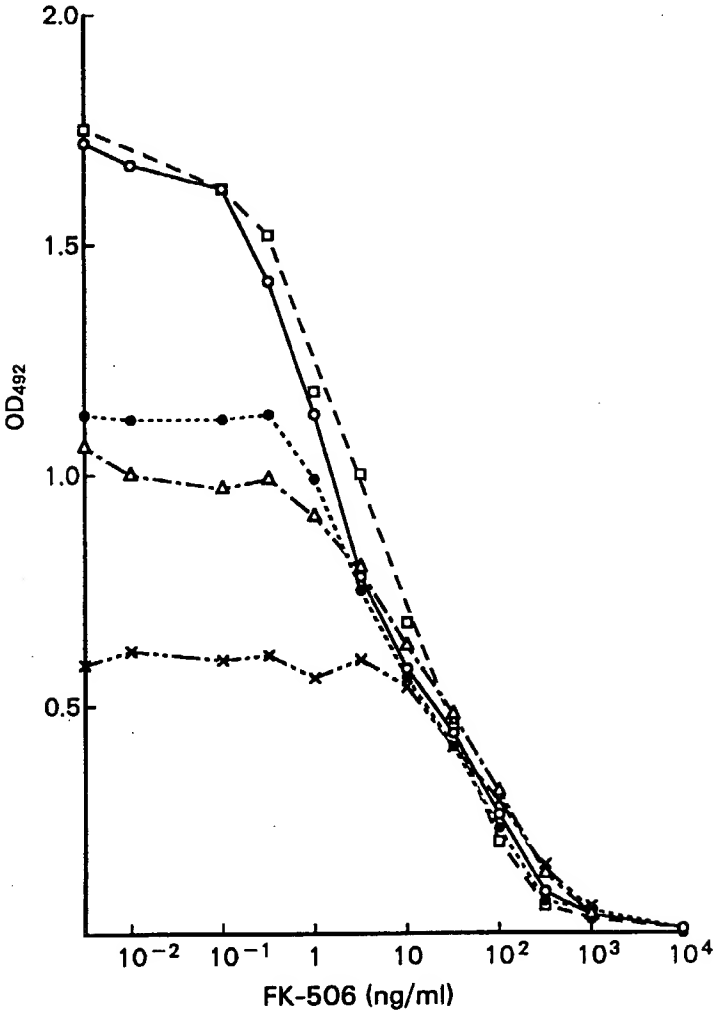


Fig 4. Influence of plasma concentration on the one-step EIA of FK-506 and elimination of the influence by benzene extraction. O—O, FK-506 in 1% BSA-PBS; ●---●, FK-506 in 10% dog plasma 1% BSA-PBS; △-·-△, FK-506 in 20% dog plasma 1% BSA-PBS; X———X, FK-506 in 50% dog plasma 1% BSA-PBS; □——□, benzene extraction from plasma.

Pharmacokinetics of FK-506 in Dogs After Oral Administration—Sensitive Two-Step EIA

The plasma concentration of FK-506 after 0.32 or 1 mg/kg administration of oral formulation (solid dispersion formulation [SDF][7]) was measured by two-step EIA after extraction pretreatment and was shown in Fig 5. In these cases, the plasma concentration of FK-506 could not be measured by one-step EIA. Oral administration gave a peak of FK-506

Table 3. Precision of MoAb Two-Step EIA: Coefficients of Variance for FK-506 in 10% Dog Plasma

	Intraassay (n = 6)		Interassay (n = 3)	
Sample	Mean ± SD (ng/mL)	CV (%)	Mean ± SD (ng/mL)	CV (%)
A	0.040 ± 0.0039	9.8	0.042 ± 0.0097	23.1
B	0.119 ± 0.0140	11.7	0.126 ± 0.0087	6.9
C	0.642 ± 0.0408	6.4	0.668 ± 0.0327	4.9
D	2.49 ± 0.1484	6.0	2.31 ± 0.3350	14.5

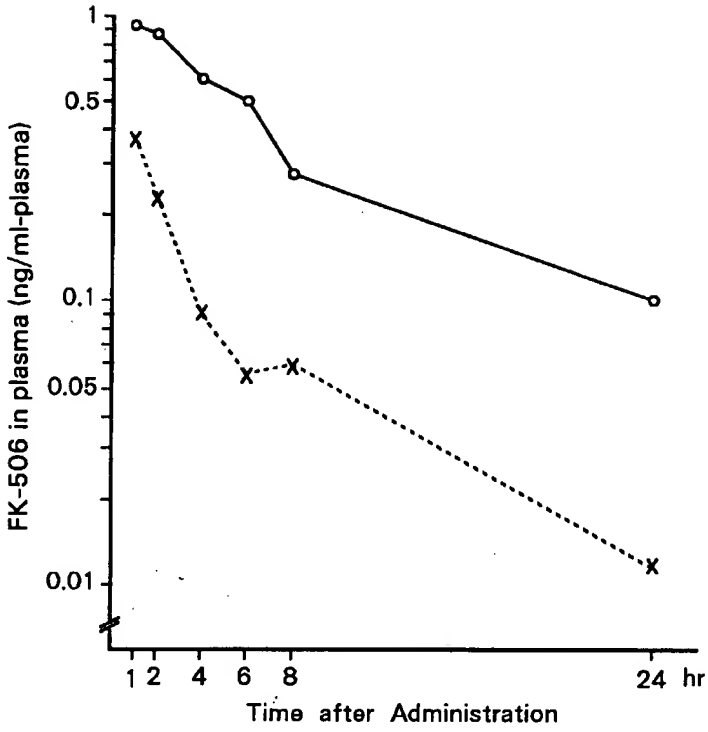


Fig 5. Pharmacokinetics of FK-506 following bolus oral administration of FK-506. X-----X, 0.32 mg/kg; O—O, 1 mg/kg.

within one hour after administration of either dosage, and at 24 hours, the FK-506 concentration was 10 pg/mL at a dose of 0.32 mg/kg and 100 pg/mL at 1 mg/kg.

DISCUSSION

Rabbit PcAb and three mouse MoAbs against FK-506 were prepared by using BSA–FK-506 as a hapten-carrier antigen. By using these antibodies, two EIA systems were established: one-step EIA and two-step EIA (Fig 2).

Anti–FK-506 Ab is adsorbed onto the plate first in one-step EIA, and the second Ab of antimouse IgG or antirabbit IgG is adsorbed onto the plate first in two-step EIA. As a result, we have four combinations of Abs and assay systems: PcAb one-step EIA, PcAb two-step EIA, MoAb one-step EIA, and MoAb two-step EIA. Of three MoAbs, MoAb 1-60-46 gave the best sensitivity in one-step EIA (Fig 3) and was chosen for further study. But even with this MoAb, the sensitivity (1 ng/mL in 10% plasma) in one-step EIA was inferior to that of PcAb (0.33 ng/mL in 10% plasma). This PcAb one-step EIA method is rather simple and has satisfactory intraassay (5.8% to 24.5%) and interassay (4.0% to 11.5%) coefficients of variance when the wide detection range of 0.33 to 10^3 ng/mL in 10% plasma was taken into account, but it was still not sensitive enough to monitor the plasma FK-506 concentration at effective doses in dogs. When the mechanism of EIA is considered, higher sensitivity can be expected with lower concentrations of POD–FK-506. But if the number of anti–FK-506 Abs adsorbed on the plate is large compared with that of POD–FK-506, the free FK-506 might occupy the extra open anti–FK-506 Ab (Fig 2). In this sense, the smaller the number of the bound anti–FK-506 Ab (either PcAb or MoAb), the higher the sensitivity of the EIA. But it is difficult to reduce the number of bound anti–

FK-506 Ab on the plate accurately by only reducing the concentration ratio of the Ab by mixing with another protein such as BSA (data not shown). Thus, we tried to lower the number of anti–FK-506 Ab bound on the plate by using a second Ab to anti–FK-506 Ab. When a combination of antirabbit IgG Ab and anti–FK-506 PcAb (rabbit) was used, the minimum detectable level rose to 1 pg/mL in 10% plasma (Fig 3). When the combination of antimouse IgG Ab and anti–FK-506 MoAb was used, the minimum detectable level rose to 20 pg/mL in 10% plasma. As is evident from Fig 3, the maximum OD_{492} was as low as 0.2 for the former combination, whereas the maximum OD_{492} for the latter combination was the normal level of 1.5. Because it was considered that, when the maximum OD_{492} is low, slight errors in reading the OD_{492} might cause a larger error in FK-506 concentration, we concluded that a combination of anti–FK-506 MoAb and antimouse IgG Ab was the one to be adopted for two-step EIA. The minimum detectable level of 20 pg/mL is a 16-fold improvement over the 0.33 ng/mL by PcAb one-step EIA and a 50-fold improvement over the 1 ng/mL of MoAb one-step EIA (Table 4).

The new method of two-step EIA afforded a highly sensitive assay method. This technique is considered not to be limited only to FK-506 but can be applied to any enzyme immunoassay that requires very high sensitivity. By using this highly sensitive EIA, we measured the plasma concentration of FK-506 after 0.32 and 1 mg/kg oral administration (SDF). As shown in Fig 5, the FK-506 plasma concentration was clearly monitored after bolus administration. With 0.32 mg/kg orally (SDF), a peak plasma concentration (0.4 ng/mL) was attained one hour after administration, and a concentration of 0.09 to 0.06 ng/mL was maintained from four to eight hours, whereas with 1 mg/kg orally (SDF), the plasma peak level was 0.9 ng/mL one hour after administration, and concentrations of 0.6 to 0.3 ng/mL were maintained from four to eight hours.

Here we have shown a highly sensitive new two-step EIA and that the pharmacokinetics of low-dose FK-506 can be monitored. The best dose form and route of FK-506 administration should be determined in accord with its pharmacokinetics.

Table 4. Summary of Minimum Detection Level

Assay	Antibody	Sample Pretreatment: Plasma Direct Method	Sample Pretreatment: Extraction Method
One-step EIA	MoAB	10 ng/mL	—
	PcAb	3.3 ng/mL	330 pg/mL
Two-step EIA	MoAb	200 pg/mL	20 pg/mL
	PcAb	10 pg/mL	1 pg/mL

Values were expressed in concentrations of dog plasma.

Fig 5. Pharmacokinetics of FK-506 following bolus oral administration of FK-506. X-----X, 0.32 mg/kg; O—O, 1 mg/kg.

this MoAb, the sensitivity (1 plasma) in one-step EIA was of PcAb (0.33 ng/mL in 10% PcAb one-step EIA method is and has satisfactory intraassay %) and interassay (4.0% to ents of variance when the wide e of 0.33 to 10^3 ng/mL in 10% ken into account, but it was still enough to monitor the plasma ntration at effective doses in e mechanism of EIA is considensitivity can be expected with rations of POD–FK-506. But if f anti–FK-506 Abs adsorbed on ge compared with that of POD–free FK-506 might occupy the iti–FK-506 Ab (Fig 2). In this aller the number of the bound Ab (either PcAb or MoAb), the nsitivity of the EIA. But it is duce the number of bound anti–

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K. TAMURA ET AL
TRANSPLANT PROC (1987) 19:23-29 (SUPPL 6).

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A single ring-enhancing lesion has been reported in 26% of CT scans in Indian patients with seizures.[2] Sethi et al[3] reported the disappearance of such lesions when anticonvulsants were prescribed, supporting a diagnosis of cerebral cysticercosis.[4] Biopsy in isolated cases has revealed tuberculosis, cysticercosis, microabscesses, and focal meningoencephalitis.[2,5] These lesions are commonly managed as tuberculomas.[2] Chandy et al,[6] however, demonstrated cysticerci in twelve of fifteen consecutive patients with seizures and single, small lesions on CT scan. There were no tuberculomas recorded. Difficulty may be encountered in differentiating cerebral tuberculomas from cysticercosis in patients from the Indian subcontinent, and these two cases suggest that measurement of CSF TBSA is unlikely to help.

I thank G. L. French, department of microbiology, Chinese University of Hong Kong (now at Guy's Hospital Medical School, London) for technical assistance.

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FK 506

SIR,—Although extensively investigated by Dr Starzl and colleagues many questions remain to be answered about the therapeutic value and safety of FK 506 in transplanted patients. The enzyme-linked immunosorbent assay, which is described by Dr Macleod and Dr Thomson (Jan 5, p 25) is non-specific for FK 506 and reacts with one or more of its metabolites (*Transplant Proc* and *Clin Biochem* in press). The trough plasma concentration (2–10 ng/ml, not mg/ml as printed) refers to a mixture of FK 506-derived compounds. The amount of drug available for immunosuppression may vary according to liver function and may not be measured accurately enough to allow therapeutic decisions to be made. An assay is required that measures specifically and sensitively FK 506 and its main metabolite(s).

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Plasma expanders as cause of paraproteinuria-like artifact

SIR,—Hydroxyethyl-starch (hetastarch, HES) preparations have been used in intensive care for more than twenty years. These polysaccharides, with molecular weights ranging from 40 to 450 kD, are eliminated predominantly by the kidneys, with half-lives up to two days.[1] 7–50 g can be found in daily urines of patients receiving 15–100 g HES, the usual dose by infusion.[1] While routinely screening for paraproteins we noted, in urine samples from patients receiving HES infusions ('Expahes', 10% HES 200/0·5 in 0·9% sodium chloride; Laevosan, Linz), paraprotein-like bands revealed by cellulose acetate electrophoresis. However, this finding could not be confirmed by immunofixation. The intensity of the narrow band near the application point depended on the concentration of HES and was demonstrable with diluted pure HES (figure, A–D). In a case of paraproteinuria (Bence-Jones proteinuria, light chain lambda) interference from HES simulated double paraproteinuria on cellulose acetate electrophoresis, not verified by immunofixation (figure, E and F).

Cellulose acetate electrophoresis (amido-black stain).

(A) Urine from patient receiving 50 g HES by infusion (glomerular proteinuria, 0·8 g/l), (B)-(D) HES in concentrations from 100 to 10 g/l, (E) urine from patient with light-chain lambda paraprotein (Bence-Jones protein), receiving HES infusion, (F) immunofixation of same urine with anti-lambda antibody.

Arrows indicate: (1) albumin, (2) light-chain lambda paraprotein, (3) HES artifact.

As an uncharged polysaccharide polymeric HES does not move on electrophoresis, but is stained blue by protein stains such as amido-black. This has also been observed for other plasma expanders such as dextrans.[2] This artifact has not received much attention so far—perhaps because of the interval between an acute infusion of HES and the later screening for paraproteins. However, in an emergency, when information about paraproteins is crucial (eg, when X-ray contrast media have to be used[3]), awareness of this artifact could be important. In such cases paraproteinuria should be sought by immunofixation.

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ALEXANDER LAPIN

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Cutaneous vasculitis and cryoglobulinaemia type II associated with hepatitis C virus infection

SIR,—We describe a patient with chronic hepatitis due to hepatitis C virus (HCV) in whom cutaneous vasculitis was associated with cryoglobulinaemia.

A 51-year-old woman presented with recurrent tender nodules over the legs, arthralgia, and Raynaud's phenomenon of 6 months' duration. The patient had been followed for the previous 4 years for non-A, non-B hepatitis. She was taking no drugs in the preceding months, and she denied parenteral drug use or known exposure to hepatitis. She had a 14 cm liver with slight tenderness and very tender red subcutaneous nodules over the lower legs. There was no purpura and her temperature was 37·4°C. Erythrocyte sedimentation rate was 20 and 47 mm/h, serum bilirubin 6 μmol/l, aspartate aminotransferase 88 IU/l, alanine aminotransferase 109 IU/l (7–29), alkaline phosphatase 152 IU/l, and latex circulating immune complexes 11·6 μg/ml (normal 0–1·5). Serum complement concentrations were normal, and mixed cryoglobulinaemia with polyclonal IgG and IgM lambda was present. IgM anti-hepatitis A and anti-hepatitis B surface antigens were not detected. Testing for HCV in serum (Ortho Diagnostics anti-HCV enzyme

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Bioassay of Plasma Specimens From Liver Transplant Patients on FK 506 Immunosuppression

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LIVER transplantation has been increasingly used to treat end-stage liver diseases since the introduction of CyA in combination with other immunosuppressive agents.[1] Although CyA has a powerful immunosuppressive effect on allograft rejection, many transplant recipients still experience rejection episodes that are often irreversible. The nephrotoxicity of CyA in some recipients has necessitated the reduction of CyA dosage below effective levels. With liver recipients refractory to conventional immunosuppression or with CyA-induced nephrotoxicity, a first-phase clinical trial of a new immunosuppressive drug, FK 506, has been initiated at our institution.[2]

FK 506 was shown by in vitro and in vivo studies to be considerably more immunosuppressive than CyA.[3-7] The successful outcome of the initial clinical studies on 14 liver transplant recipients[2] has led to a formal clinical trial of FK 506 as the primary immunosuppressive agent in combination with low doses of steroids.[8]

Appropriate dosages of FK 506 are being determined to provide optimal immunosuppression without significant adverse side effects. The monitoring of blood levels of FK 506 is essential in these studies. This monitoring has become possible with an enzyme immunoassay (ELISA) using anti-FK 506 MoAbs. This assay was developed by Tamura et al[9] and modified by Cadoff et al.[10]

FK 506 is a highly lipophilic macrolide (molecular weight 822).[11] Although little is known about the metabolites of FK 506, recent studies have implicated the liver as the primary site of FK 506 metabolism.[12]

The objective of this study was to determine the relationship between plasma levels of FK 506 as measured by ELISA and by a bioassay based on the proliferative response of an alloreactive T cell line.

MATERIALS AND METHODS

Plasma

Five milliliters of heparinized blood was collected from four liver transplant recipients during the first month following transplantation. Each sample was drawn just prior to FK 506 administration. All blood samples were incubated at 37°C for 1 hour prior to centrifugation. The plasma was analyzed for FK 506 trough levels using an indirect ELISA[10] and a bioassay described in detail below.

Drug Source

FK 506 was supplied in crystalline form by the Fujisawa Pharmaceutical Co Ltd, Osaka, Japan. A stock solution of 100 μg/ml FK 506 was prepared in methanol and kept at −4°C.

Bioassay of FK 506 Levels in Plasma

Inhibition Effect of FK 506 on Lymphocyte Proliferation. This was determined in a secondary proliferation assay using a DQw1-specific alloreactive T cell clone DB29[13] as responder cells. Frozen cloned cells were thawed and washed in RPMI 1640 (Gibco) and resuspended at 10^5 cells/ml in tissue culture medium (TCM) consisting of RPMI 1640 supplemented with 25 mmol/L Hepes buffer, 100 U/ml gentamicin, and 10% nontransfused human male serum. The inhibitory effect of FK 506 on the primed lymphocyte (PLT) response of DB29 cells was measured at different concentrations of the drug, ranging from 0.003-1 ng/ml. In these 3-day PLT assays, 10^4 responder cells were incubated in triplicate with 10^5 DQw1-positive stimulator cells irradiated (2,000 rad) in 200 μl of TCM. During the final 20 hours of incubation, each culture was labeled with 1 μCi of ^{3}H-thymidine. The cultures were harvested and counted in a liquid scintillation counter (LKB). The inhibitory effect of FK 506 at various concentrations was determined with the following formula:

$$\text{percent inhibition} = \left(1 - \frac{\text{cpm with drug}}{\text{cpm without drug}}\right) \times 100$$

Effect of Plasma Samples on Lymphocyte Proliferation. Plasma samples were diluted (½ to 1/64) in TCM. The inhibitory effect of different plasma dilutions on the PLT response of DB29 cells was measured as described for FK 506. In these assays, 10^4 responder cells (50 μl) and 10^5 stimulator cells (50 μl) were incubated with 100 μl of plasma. A pretransplant plasma sample from each patient was used as a control. The results were calculated with the following formula:

$$\text{percent inhibition in plasma} = \left(1 - \frac{\text{cpm with test plasma}}{\text{cpm control plasma}}\right) \times 100$$

Calculation of FK 506 Levels From Bioassay Results. The plasma concentrations of FK 506 were calculated from the inhibition curve observed with various plasma dilutions in comparison with a standard curve for FK 506. These calculations were made using a nonlinear sigmoidal model curve fitting program (Statistical Analysis System [SAS] Institute, release 5.18, Cary, NC). The

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IC_{50} from the standard curve was determined as the concentration of FK 506 (ng/ml) which caused 50% inhibition of the PLT response of DB29. From the plasma inhibition curve, the ID_{50} was determined as the dilution factor for which plasma induced 50% inhibition of PLT response of DB29. From these values, the plasma concentration of FK 506 was calculated as $IC_{50} \times ID_{50}$.

RESULTS

Initial studies showed highly consistent dose response curves of PLT inhibition of DB29 cells by FK 506. Fig 1 shows representative results of three separate experiments. These data yielded a mean IC_{50} value of 0.063 ± 0.001 ng/ml.

Examples of PLT inhibition of DB29 by patient plasma are shown in Table 1. These bioassays were done on a liver allograft recipient at 7, 12, and 14 days posttransplant. The plasma FK 506 levels were calculated by multiplying the ID_{50} values with the IC_{50} value of 0.063 ng/ml.

Comparison Between FK 506 Levels Measured by ELISA and Bioassay

In all plasma samples tested, the values for FK 506 levels obtained by ELISA were generally much higher than those determined in the bioassay. Fig 2 compares FK 506 levels determined by ELISA and bioassay for two liver transplant recipients.

Patient P2 was originally diagnosed with sclerosing cholangitis and had lost five previous liver allografts while on CyA treatment. Following the sixth transplant, 6 mg/d of FK 506 was administered intravenously for 7 days. Methylprednisolone (1 g) was given on the second day posttransplant and was tapered daily to a maintenance dose of 10-20 mg/d. Although FK 506 levels determined by bioassay were always lower than those measured in ELISA (0.2-2 ng/ml versus 0.5-4.5 ng/ml), they showed a significant correlation ($P < 0.001$, $r^2 = 0.77$).

Following the first bolus of methylprednisolone (day 2 postsurgery), the FK 506 concentrations measured by ELISA were four- to fivefold higher than the bioassay levels. FK 506 was discontinued for 4 days; the ELISA concentrations of FK 506 dropped sharply to 0.5-1 ng/ml, and no detectable immunosuppressive activity was found in the bioassay in those plasma samples. On day 11 postsurgery, the patient showed clinical symptoms of graft dysfunction as determined by changes in liver enzymes. On the same day, FK 506 (6 mg/d) and a bolus of methylprednisolone (1 g) were administered. Consequently, there was a rapid increase in the ELISA plasma concentrations of FK 506 and a more gradual rise in the bioassay-determined FK 506 levels (Fig 2a).

Similar findings are shown for patient P14, who was

Table 1. Bioassay of FK 506 Levels in Plasma From a Liver Transplant Recipient

Days Posttransplant	Dilutions (% Inhibition) 1:4	1:8	1:16	1:32	ID_{50}*	FK 506† (ng/ml)
7	99	89	48	0	15	0.94
12	93	40	0	0	7	0.44
14	99	98	76	16	21	1.32

*ID_{50} represents the plasma dilution factor causing 50% inhibition of PLT response of DB29 cells.
†Calculated by multiplying ID_{50} by the IC_{50} of 0.063 ng/ml.

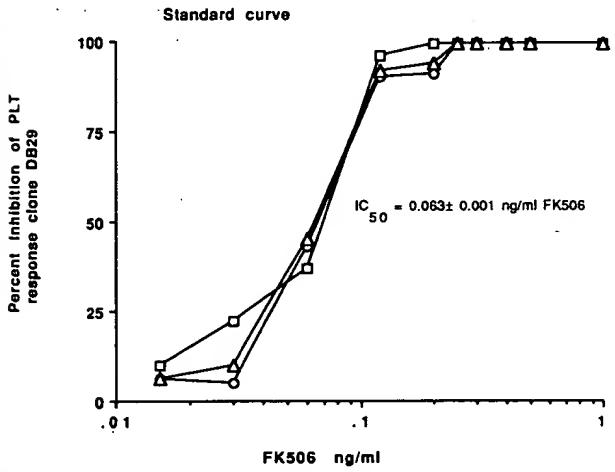

Fig 1. Standard curves of the FK 506 bioassay. The concentration of FK 506 is plotted versus percent inhibition of PLT response of DB29 cells. The IC_{50} was calculated using a nonlinear sigmoidal model fitted to the points.

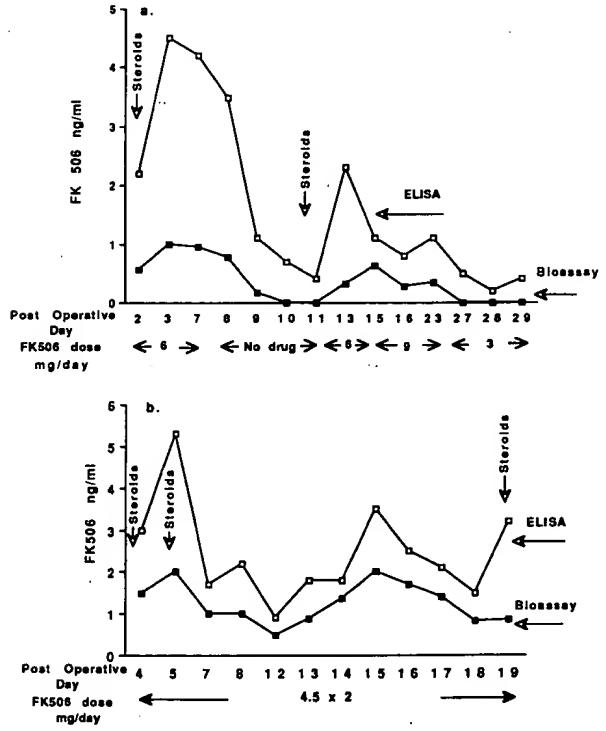

Fig 2. FK 506 levels in plasma measured by the bioassay and by the ELISA for liver transplant patients. **(a)** P2 and **(b)** P14.

diagnosed with hepatitis B and juvenile diabetes mellitus. He received kidney, pancreas, and liver allografts. FK 506 (4.5 mg) was administered intravenously two times per day, and this dose was maintained for 19 days posttransplantation. Steroids were given on days 2, 5, and 19 postsurgery.

A significant correlation ($P < 0.001$, $r^2 = 0.75$) was found between the two assays for plasma levels of FK 506 (Fig 2b).

Following steroid administration, FK 506 levels determined by bioassay were still much lower than ELISA plasma concentrations of FK 506.

Relationship Between FK 506 Dose and the Plasma Concentration

There was no apparent correlation between the dose of FK 506 given and the concentration of FK 506 in the patient's plasma. Although patients P14, P16, and P32 received the same dose of FK 506, the plasma levels of FK 506 (ELISA and bioassay) were much higher in patients P14 and P16 than in the patient P32 (Fig 2 and Table 2). Furthermore, in three of four plasma samples tested from patient P32, the bioassay levels of FK 506 were <0.1 ng/ml, whereas the ELISA values ranged from 0.3-1.6 ng/ml. This patient had a histologically confirmed cellular rejection. Patient P16, on a similar dose of FK 506, had a stable posttransplant course. His plasma concentrations of FK 506 as determined by ELISA and bioassay were 1.2-6 ng/ml and 1-4 ng/ml, respectively.

DISCUSSION

This study was designed to assess the correlation between FK 506 levels measured by bioassay and ELISA.

The bioassay was based on the inhibition of PLT reactivity of an alloreactive T cell clone. Low concentrations of FK 506 significantly inhibited the proliferative responses of mixed lymphocyte reaction (MLR)-derived alloreactive T cell clones,[4] and this inhibition was reproducible when the same T cell line was used (Fig 1). The sensitivity of the ELISA for FK 506 was 0.1 ng/ml[10] whereas in the bioassay, drug levels of 0.063 ± 0.001 ng/ml had a significant inhibitory effect on the responder cells.

Table 2. Correlation Between Bioassay and ELISA

Days Posttransplant	Drug Dose (mg/day)	Bioassay (ng/ml)	ELISA (ng/ml)
Patient P32			
2	4.5 × 2 IV*	< 0.1	1.6
5	4.5 × 2 IV*	1.7	6.4
8	9 × 2 PO†	< 0.06	0.9
12	9 × 2 PO†	< 0.06	0.3
Patient P16			
2	9 × 2 PO†	2.4	2.6
3	9 × 2 PO†	1.7	2.3
4	9 × 2 PO†	1.0	1.2
5	9 × 2 PO†	1.3	1.8
6	9 × 2 PO†	> 4	6
7	9 × 2 PO†	2.5	3

*Intravenous dose of 4.5 mg FK 506 twice a day.
†Oral dose of 9 mg/d twice a day.

The levels of FK 506 determined in bioassay were consistently lower than those measured by ELISA. This suggests that the blood may contain inactive metabolites of FK 506 which could be detected by the ELISA. Since the ELISA is an immunoassay, it may measure biologically active and non-active metabolites that might cross-react with the anti-FK 506 antibody. Similarly, CyA metabolites are measured by monoclonal and polyclonal radioimmunoassay (RIA)used for monitoring trough levels of CyA in transplant recipients.[14]

FK 506-treated patients received a bolus of high-dose steroids the second day posttransplant and when acute allograft rejection was suspected. Steroids could affect the determination of FK 506 levels by the bioassay since this assay does not discriminate between various immunosuppressive drugs. Nevertheless, the FK 506 levels measured by bioassay poststeroid treatment were not much affected in contrast to the marked increases in immunoassayed FK 506. It is unclear how steroids influence ELISA assay. Corticosteroids have been shown to be inducers of hepatic drug metabolism.[12] Experience with CyA immunosuppression has demonstrated that RIA levels of CyA increased after steroid treatment while high-pressure liquid chromatography-measured CyA levels appeared unaffected.[15] Further studies are needed to elucidate the potential effect of steroids on FK 506 metabolism.

Biologic assays have previously been used for the measurement of CyA in patient's plasma based on the inhibition by the plasma of a third-party MLR. No attempt was made, however, to express the inhibitory activity as a concentration of biologically active CyA. Both Kahan[14] and Reisman et al[16] suggested that biologic assay has clinical relevance; patients with continually low plasma immunosuppressive activity are prone to rejection of their kidney allografts. Our preliminary observations also indicated that the absence of immunosuppressive activity in patient's plasma was associated with liver allograft rejection (patient P32).

Recently, Jain et al[17] have shown that moderate to severe hepatic dysfunction induced an increase in trough levels of FK 506 measured by ELISA. Also, the CyA RIA levels were higher than the specific measurements of CyA during rejection episodes following liver transplantation.[18] Studies are currently in progress to analyze the relationship between ELISA and bioassay levels during acute allograft rejection.

In conclusion, a bioassay based on the inhibition of an alloreactive T cell clone proliferation was used to determine FK 506 levels in patient's plasma. A positive correlation was demonstrated between drug levels measured by the bioassay and ELISA. However, the FK 506 levels were lower for the bioassay than for ELISA. Since FK 506

cannot yet be assayed accurately in plasma using specific chromatographic techniques, the bioassay is important for assisting in the interpretation of an immunoassay (ELISA) with unknown specificity for biologically non-active forms of FK 506. Furthermore, biologic assay may be useful for patient management if active metabolites are induced, or to detect the interaction between FK 506 and other drugs (high-dose steroids) in the immunosuppression of allograft rejection.

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Assay of FK 506 in Plasma

E.M. Cadoff, R. Venkataramanan, A. Krajack, A.S. Jain, J.J. Fung, S. Todo, and T.E. Starzl

FK 506 is a potent immunosuppressive agent in vitro and in vivo in animals and, as discussed extensively in this symposium, in humans. In contrast to the early use of CyA, an assay for the measurement of FK 506 has been available for use in these early studies.[1] We present our experience with the modified enzyme immunoassay (EIA) procedure we have used in preclinical studies and in the early clinical trials of FK 506.

MATERIALS AND METHODS

FK 506 Assay

The FK 506 assay is an EIA using a mouse monoclonal anti-FK 506 antibody (Fujisawa Pharmaceuticals Co Ltd, Osaka, Japan). Briefly, anti-mouse IgG is adsorbed onto a 96-well flat-bottomed microtiter plate (Flow Labs, McLean, VA) overnight at 4°C and the plate is blocked with 0.5% albumin. FK 506 (Fujisawa) standards (a zero standard and from 0.1 to 5.0 ng/ml) are prepared 10× concentrated in methanol (MeOH). One hundred microliters of plasma is pretreated with 1 ml of 0.1 N HCl, and 100 μl of FK 506-free plasma is used for the standards. Ten microliters of standard solution or MeOH blank is then added to each sample. One milliliter of the treated sample is loaded onto a C-18 mini-column (Sep-pak, Waters, Milford, MA) that has been prewet with MeOH and acetic acid. The samples are washed with acetic acid and eluted with 3.0 ml of MeOH. The eluate is evaporated to dryness and reconstituted with peroxidase labeled FK 506 (Fujisawa). Reconstituted sample and monoclonal antibody are placed in each well, and competitive binding occurs overnight at 4°C with gentle agitation. Unbound FK 506 is removed, and the activity of the bound FK 506-peroxidase conjugate is measured by the increase in optical density (OD) at 492 nm after a 20-minute incubation with o-phenylenediamine (Sigma, St Louis, MO) substrate.

The standard curve is linearized using a log-logit transformation, and controls and unknowns are calculated from the regression line.

Precision, Accuracy, and Recovery Studies

Pools of human plasma were spiked with FK 506 to concentrations of 0.5, 1.0, and 2.0 ng/ml. Aliquots of these samples were taken with each run to determine accuracy and precision. Recovery of the extraction procedure was assessed by diluting 10 μl of the standards to 3 ml in MeOH and processing them further as mini-column eluates.

Interference

Samples with a variety of bilirubin and FK 506 concentrations were prepared using normal plasma and plasma from a jaundiced patient not on FK 506; the plasma was spiked with FK 506. A similar matrix was created with plasma contaminated with hemolyzed red blood cells. FK 506 was measured in samples sent for CyA determination to look for cross-reactivity.

Separation of Plasma

Whole blood samples from patients on FK 506 are mixed thoroughly and divided into two aliquots. One is left at room temperature and centrifuged, the second is incubated at 37°C for a minimum of 30 minutes, then spun at 37°C. Plasma from both aliquots is stored at 4°C until assayed.

RESULTS

FK 506 Enzyme Immunoassay

Measurements of OD vary considerably from day to day, with the zero standard ranging from 0.8 to 2.0. However, the variation is consistent within each run. The coefficient of variation (CV) of the FK 506 assay is 27% at 0.5 ng/ml, 23% at 1.0 ng/ml, and 15% at 2.0 ng/ml. The assayed values of these samples are 0.47, 0.93, and 2.1 ng/ml, respectively, with accuracies thus ranging from 93% to 95%. The recovery of the extraction ranges from 100% at 0.5 ng/ml to 125% at 5.0 ng/ml. The linear range is 0.1 to 5.0 ng/ml. Samples with higher concentration are diluted with FK 506-free plasma.

Interference Studies

Hemolysis does not interfere with the FK 506 assay, even when marked hemolysis is present. A low FK 506 concentration sample measured 0.1, 0.1, and < 0.1 ng/ml when slight, moderate, or marked hemolysis was present. Another measured 0.5, 0.3, and 0.3, respectively, and a third measured 1.1, 1.0, and 1.2. Similarly, at bilirubin concentrations of 10, 160, and 310 μmol/L (0.5, 9, and 18 mg/dl), FK 506 measured < 0.1, < 0.1, < 0.1, respectively, in one sample, 0.5, 0.4, and 0.4 in a second, and 1.7, 1.7, and 1.9 in a third. CyA concentrations of up to 2,000 ng/ml by FPIA (TDx^R) did not cross-react in the FK 506 assay, reading < 0.1 ng/ml FK 506.

Plasma Separation

For 220 samples from eight patients, separation of plasma at both room temperature and at 37°C was done. Plasma separated at room temperature contained <75% of the

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amount of FK 506 compared with its 37°C aliquot (room temperature = 0.75 × [37°C] − 0.2; $r = 0.9763$).

Dose Response

In 30 patients, the relationship between dose of FK 506 and 12-hour trough concentrations was studied with a correlation coefficient of $r < 0.3$. To eliminate the interpatient variability, one patient was identified in whom an intrapatient dose-response curve could be examined. Regression revealed a correlation coefficient of $r < 0.33$ in this patient.

DISCUSSION

The FK 506 assay has performed satisfactorily in our laboratory. Measurement of FK 506 concentrations in animal studies has shown wide interspecies variation in kinetics[2] and dosing requirements,[3] and in vitro studies have shown interspecies variations in sensitivity of lymphocytes to FK 506.[4] It was therefore felt that the assay system would be necessary for adjusting dosing and determining pharmacokinetics in phase I clinical trials.

Although the OD varies from day to day, we were unable to discern either a consistent pattern or the reason for this variation. Despite this effect, the shape of the standard curve is uniform each day, and results of the control samples are consistent. The 15-27% CV compares reasonably with that of CyA—our current CyA high-pressure liquid chromatography assay has a CV of 14-22% and our radioimmunoassay has a CV of 10-19%. Automation of CyA for the TDx^R has improved the CV to 7-14%. Two major factors accounting for the lesser precision of the FK 506 assay are the low concentrations of the agent and the inexactness of an EIA. The calculated accuracy and recoveries are not significantly different from 100%.

Since FK 506 kinetics differ in patients with liver failure,[5] it was essential to demonstrate that this is not due to bilirubin interfering with the assay. Similarly, FK 506 measurements on hemolyzed samples are accurate.

Initial pharmacokinetic studies in humans and experience with CyA have suggested that the distribution of FK 506 between red blood cells and plasma may be temperature-dependent. Indeed, we did find such a temperature dependence and have switched to 37°C separation to reflect in vivo conditions. This has allowed for reliable studies on the distribution and elimination of FK 506.[6]

The poor correlation between dose and plasma concentration of FK 506 is similar to that seen in baboons.[3] However, the safety and efficacy profile seen in the phase I clinical trials may obviate the need for precise and frequent measurement of FK 506 to fine-tune plasma concentrations.[7-9]

The current FK 506 assay has been essential to the preclinical and early clinical trials of FK 506, and measurement of FK 506 will be necessary as clinical trials continue. Measurement of FK 506 concentrations will be necessary early in therapy, especially for patients with liver failure, but routine "FK 506 levels" may not be needed as frequently as they are for CyA.

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Monitoring FK 506 Concentrations in Plasma and Whole Blood

W.J. Jusko and R. D'Ambrosio

THE immunosuppressive agent FK 506 is undergoing clinical trials for transplantation of the liver and other organs. This drug is extensively metabolized by the liver, has limited and erratic bioavailability because of its poor water solubility and appreciable first-pass effect, and is susceptible to fluctuations in plasma concentrations because of the changing pathophysiology of transplant patients that further complicates FK 506 absorption and disposition.[1,2] Plasma and whole blood monitoring of FK 506 was called for because of these factors, the general experience with cyclosporine (CyA),[3] and the desirability to identify a possible therapeutic range for patient monitoring.

The FK 506 Central Laboratory (FCL) was established at the State University of New York at Buffalo in May 1990 for the initial purposes of adapting and partly validating the enzyme immunoassay (EIA) assay for FK 506[4,5] and to provide plasma and whole blood monitoring of patients undergoing liver transplantation at clinical sites in the United States outside of Pittsburgh. Other analytical laboratories were established in Japan and Europe. Later, the mission of the FCL was expanded to assist other laboratories in establishing the same assay procedure and to evolve a quality assurance program to seek equivalent assay results for all collaborating clinical sites.

This report will describe our results in using the modified EIA assay for FK 506 for plasma and whole blood, some experiences in patient monitoring particularly the relationship between whole blood and plasma concentrations, and initial functioning of the interlaboratory quality assurance program.

MATERIALS AND METHODS

The FK 506 EIA assay employs a mouse monoclonal anti-FK 506 antibody (Lot #B01005S, Fujisawa Pharmaceutical Co, Osaka, Japan). Goat antimouse IgG (Atlantic Antibodies) is adsorbed onto a 96-well flat-bottomed microtiter plate (ICN Flow Inc, or Nunc Inc) overnight at 4°C and the plate is then blocked with 1% bovine serum albumin (BSA) and 0.05% Tween-20 in phosphate buffer (BTPBS). The FK 506 (Lot #010186L, Fujisawa) standards are initially prepared and diluted in methanol before dilution into plasma or heparinized whole blood. The plasma (300 μL) or whole blood (20 μL) standards and samples are added to 1 mL of pH 7.4 phosphate buffer in Teflon-lined, screw-capped, glass culture tubes and FK 506 is extracted into 9 mL of methylene chloride by shaking for 30 minutes. After centrifugation at 4°C and removal of the upper aqueous phase, 7.5 mL of methylene chloride is transferred to a glass tube and evaporated to dryness using N_2 gas. The samples are reconstituted with 450 μL of 1% BSA and 0.05% Tween-20 in phosphate buffer. This solution (140 μL) is placed into duplicate microtiter wells followed by 40 μL of the FK 506-peroxidase enzyme conjugate (Fujisawa Lot #B010935) diluted in BTPBS and by 50 μL of the diluted anti-FK 506 antibody. The peripheral wells of the plate are not used in this procedure. The plate is sealed and incubated overnight at 4°C in a humidified chamber (12 to 18 hours). The wells are aspirated, washed four times, and freshly prepared O-phenylenediamine HCl (Cat. No. 88412, Sigma, St Louis, MO) solution is added to each well. After agitation for 15 minutes in the dark, the reaction is halted with 50 μL of 4 N H_2SO_4. The plates are shaken and the color is read at 490 nm using a Biotek 2000 Kinetics Elisa Reader (Fisher Scientific). Various steps in the enzyme reaction procedure are done sequentially by rows of wells and precisely timed.

The KinetiCalc 2.026 (Fisher Scientific) software program is used to fit the absorbance (F) vs FK 506 concentration (X) data to the four-parameter logistic curve.

$$F = D + \frac{(A-D)}{1 + \left(\frac{X}{C}\right)^{B}} \quad (1)$$

where the following are the fitted parameters: A is the expected response at zero analyte concentration; B is the slope factor or exponent; C is the EC_{50}, ie, analyte concentration with an expected response half-way between A and D; and D is the expected response for infinite analyte concentration.

Conventional validation procedures[6,7] were used to assess the sensitivity, low limit of quantitation (LOQ), high limit of quantitation (HOQ), and within-day and between-day coefficients of variation (CV) of the assay for FK 506 in plasma and whole blood. The assay was applied in the analysis of several thousand plasma and whole blood samples from patients undergoing liver transplantation.

RESULTS AND DISCUSSION

Assay Performance

Standard curves for FK 506 in plasma and whole blood are shown in Fig 1. This is typical of the EIA results with good agreement between the actual and predicted drug concentrations (based on Eq 1), reasonable standard deviation of duplicates, and an overall curve-fitting with $r^2 \geq 0.99$. Table 1 provides the sensitivity, LOQ, HOQ, and CV of the assay in analysis of prepared standards. The assay functions reasonably well for an immunoassay measuring relatively low concentrations of analyte (LOQ of 0.2 ng/mL in plasma). The standard curves were designed with

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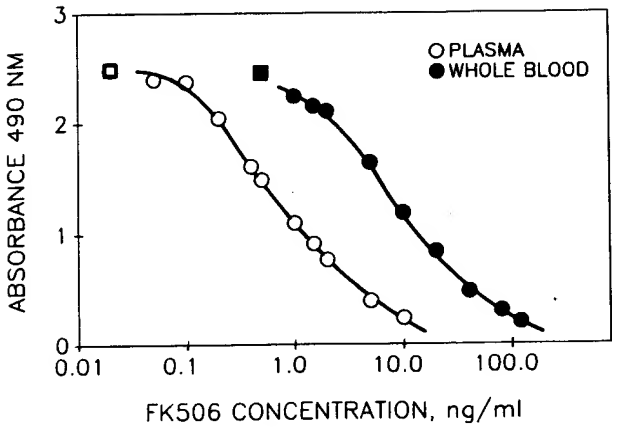

Fig 1. Typical standard curves for FK 506 EIA in plasma (○) and whole blood (●) with fitting using Eq 1. Squares denote absorbances for 0 FK 506 concentrations. See Table 1 for the characteristics of the assay in analysis of standards in 0.3 mL plasma and 20 μL whole blood.

the expectation that the therapeutic range of FK 506 in plasma would be bracketed by 0.5 to 2.0 ng/mL concentrations. Whole blood concentrations are 20 to 30 times higher. Typical values of the fitted parameter C (or EC_{50}) for plasma are 0.84 ± 0.17 ng/mL and for whole blood are 5.3 ± 2.4 ng/mL. The actual absorbances for 0 FK 506 standards usually range from 1.0 to 2.5 absorbance units (AU) from run to run. Samples that produce nonspecific binding (infinite FK 506 concentration) yield absorbances that average about 0.060 AU, well below and statistically different from the highest FK 506 standards.

The daily assay procedure calls for measuring two each of low (0.3 ng/mL plasma, 4 ng/mL whole blood), medium (1.2 ng/mL plasma, 15 ng/mL whole blood), and high (3.0 ng/mL plasma, 60 ng/mL whole blood) quality control samples placed on each microtiter plate. No more than two different FK 506 concentrations are allowed to yield results outside the limits of ±30%. Replication of 10 samples at specific FK 506 concentrations on the same plate yields CV of absorbances that range betwen 3.6% and 7.5%. This appears to be the minimally attainable precision of the present assay. The use of Nunc vs ICN Flow microtiter plates yields comparable CV for repeated analysis of quality control (QC) samples.

Figure 2 shows the results of the plasma assays over 21 days of analysis along with the interday CV that ranged from 13% to 23%. When errant values occur with the QC samples, the assay tends to produce high values.

Table 1. Characteristics of the Assay in Analysis of Standards in 0.3 mL Plasma and 20 μL Whole Blood

	Plasma	Whole Blood
Sensitivity	0.1	0.7 ng/mL
LOQ	0.1–0.2	1.0 ng/mL
HOQ	>10	>120 ng/mL
CV	4%–27%	4%–17%

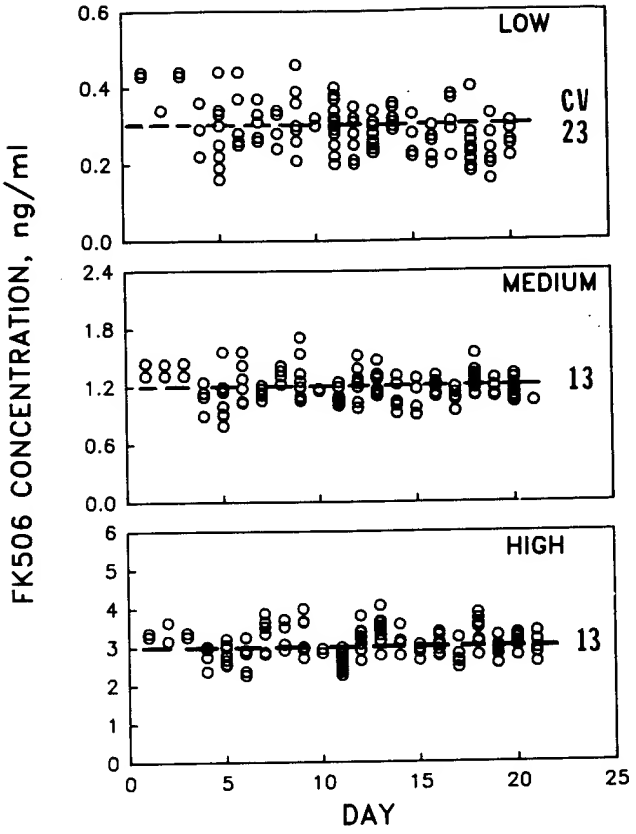

Fig 2. Interday variability in analysis of QC plasma samples containing three concentrations of FK 506. CVs are indicated for each panel.

The main difference between the present and previous[2] EIA assays of FK 506 is the use of methylene chloride (MeCl) vs Sep-Pak solid extraction systems. An early cross-check of the comparability of results showed no more than a 30% difference between the methods. The use of methylene chloride liquid extraction allows more samples to be processed per day by each technician. This is largely due to shorter evaporation time.

Patient Monitoring

The use of FK 506 for immunosuppression of patients undergoing liver transplantation in protocol FPC-FK 506-7 (Fujisawa) currently calls for doses of 0.05 mg/kg infused over each 12-hour period for the first 3 days followed by oral doses of 0.15 mg/kg every 12 hours thereafter. Dosage adjustments are made based on patient responses, adverse effects, and a general guideline of the desirability of maintaining plasma concentrations between 0.2 and 2.0 ng/mL. Blood samples are collected to represent trough values (12 hours after dosing) and plasma is separated from whole blood by incubation and centrifugation at 37°C.

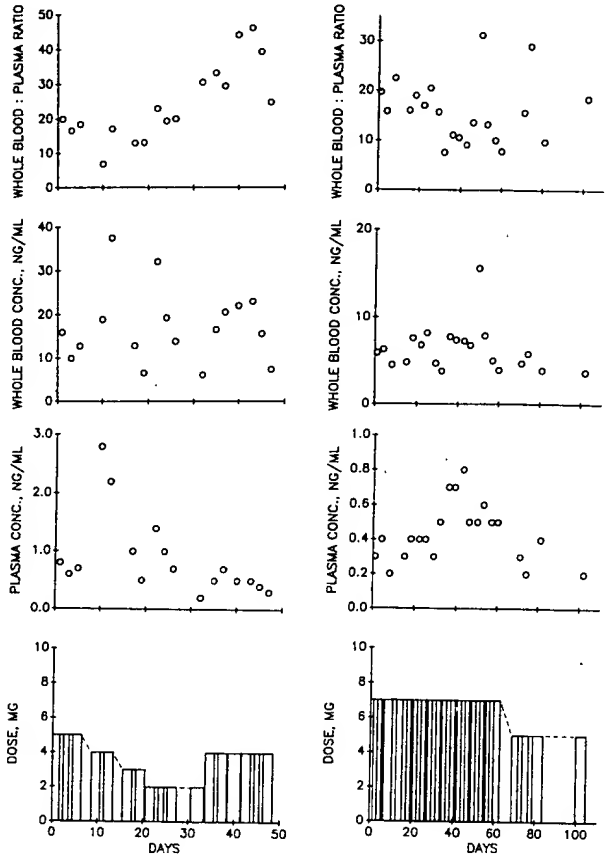

Fig 3. Representative profiles of FK 506 dosage regimen (mg/12 h), plasma concentrations, whole blood concentrations, and WBPR for two patients.

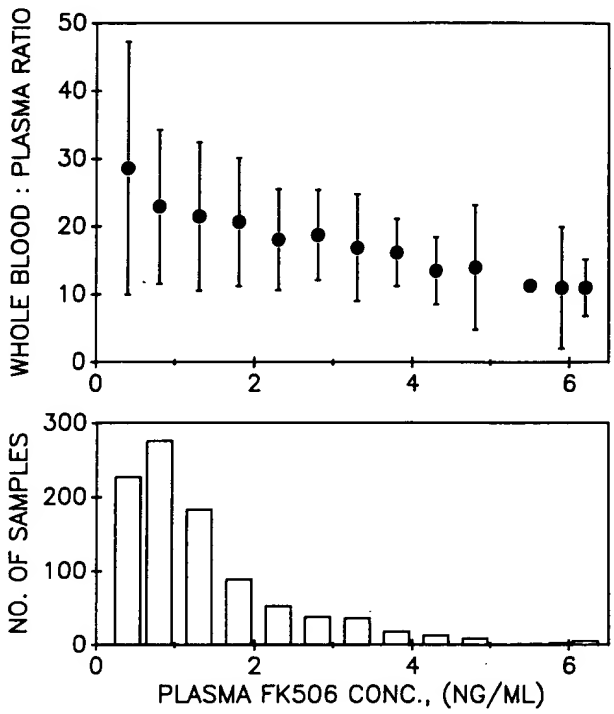

Fig 4. Distribution of plasma FK 506 concentrations found in 1,000 consecutive liver transplant patient samples along with the WBPR (mean ± SD). An additional 50 samples that had FK 506 concentrations <0.2 ng/mL are not depicted.

Whole blood to plasma ratios (WBPR) are known to be temperature dependent with lower temperatures causing increased red cell binding.[2]

Figure 3 shows typical profiles of the dosage regimen, plasma and whole blood FK 506 concentrations, and the WBPR for two patients. Plasma FK 506 concentrations were initially low, showed gradual accumulation, and changed with dosage. Whole blood concentrations tended to be more stable. The WBPR was about 20 in the beginning and changed gradually as plasma concentrations increased or decreased.

Our experience in monitoring 1,000 consecutive plasma and whole blood samples from patients on the liver transplant protocol is reflected in Fig 4. Fifty samples assayed below the plasma LOQ (<0.2 ng/mL) and are not shown. Further, 77.5% of the plasma concentrations fell in the primary target range of 0.2 to 2.0 ng/mL. Only 0.8% of the samples exceeded 5.0 ng/mL.

The summary data in Fig 4 depict the nonlinear relationship between whole blood and plasma concentrations of FK 506. The WBPR averages 29 ± 19 at low FK 506 plasma concentrations (0.3 to 0.5 ng/mL) and decreases to 11.0 ± 9.0 at concentrations of 6 ng/mL. While FK 506 is more easily measured in whole blood because of its higher concentration, the nonlinear WBPR may confound the interchangability of plasma and whole blood analyses for therapeutic monitoring. It is as yet uncertain which biologic medium best reflects the equilibrium of FK 506 with its sites of action in lymphocytes.[8] CyA also exhibits temperature and concentration dependence in red cell to plasma distribution.[9] However, the nonlinearity occurs below the therapeutic range, which does not complicate monitoring plasma vs whole blood concentrations of this drug.

Quality Assurance Program

The initial use of the FK 506 assay outside of Pittsburgh involved the services of the FCL as the primary analytical site in the United States. Samples were shipped to Buffalo by overnight express and the EIA assays were performed with a 3-day turnaround. Subsequently, the plasma and/or whole blood assays have been set up at 12 additional sites for more rapid patient monitoring.

The quality assurance program, which has been set up to seek consistency of data among sites, consists of the following: (1) an initial partial validation program involving evaluation of the functioning of the reagents and equipment and demonstration of satisfactory intra- and interassay precision and accuracy; (2) the analysis by each site of

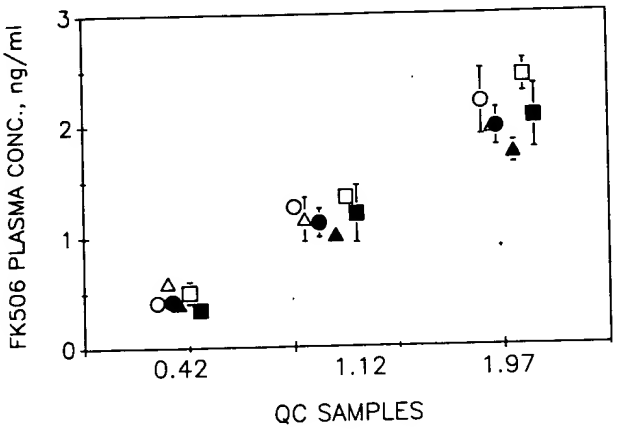

Fig 5. Attainment of designated FK 506 concentrations in a portion of the plasma "known-unknowns" by the FCL (●) and by five other analytical laboratories during initial phases of the quality assurance program.

a set of blinded samples with satisfactory performance being a 70% successful assay of 30 samples to within ±30% of the predetermined FK 506 concentration; (3) monthly exchange of FK 506 samples with three known-unknowns disseminated by the FCL and six random patient samples returned from the clinical sites to the FCL for reanalysis. The FCL also maintains drug and antibody reagents provided by Fujisawa in Osaka, Japan, for replenishment of the supplies of other laboratories in the United States.

An indication of the close correspondence and high precision of the FCL and the first five laboratories operating the FK 506 plasma assay is provided in Fig 5. All sites achieved very similar analysis of these three concentrations of FK 506 found in part of the batch of "known-unknowns" provided for assay.

SUMMARY

The FCL provides support for clinical trials of FK 506 in liver transplantation by monitoring drug concentrations in plasma and whole blood. The sensitive EIA method of Tamura et al[4] was adapted for routine use. Extraction of drug from 300 μL plasma or 20 μL whole blood samples was carried out using MeCl. An alternative solid phase (Sep-Pak) extraction method[5] was also tested. Nonlinear temperature-sensitive binding of FK 506 to red cells necessitates plasma separation from whole blood at 37°C. The analytical procedure entails numerous steps and efforts were made to improve the accuracy of the method. Interday CVs of FK 506 in plasma (0.3 to 3.0 ng/mL) and whole blood (4 to 60 ng/mL) ranged up to about 20% during validation testing and are maintained below 30% during routine assay use. However, daily QC samples occasionally yield unexpectedly high FK 506 concentrations. The MeCl and solid phase extraction methods yield FK 506 concentrations that reasonably correlate but the MeCl method produces plasma concentrations that are about 30% lower. FK 506 is appreciably bound in red cells with a nonlinear WBPR of 20 to 50 at low plasma concentrations (0 to 2 ng/mL) and a ratio of about 11 at plasma concentrations above 5 ng/mL. This may complicate conversion from plasma to whole blood for routine therapeutic monitoring. The described procedures for FK 506 are being implemented at various clinical sites in the United States with the FCL providing assistance with a quality assurance program to assure intersite comparability of assay results.

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mixing the two materials. "While the references do not show a specific recognition of that result, its discovery by appellants is tantamount only to finding a property in the old composition." 363 F.2d at 934, 150 USPQ at 628 (emphasis in original).).

2113 **Product-by-Process Claims**

PRODUCT-BY-PROCESS CLAIMS ARE NOT LIMITED TO THE MANIPULATIONS OF THE RECITED STEPS, ONLY THE STRUCTURE IMPLIED BY THE STEPS

"[E]ven though product-by-process claims are limited by and defined by the process, determination of patentability is based on the product itself. The patentability of a product does not depend on its method of production. If the product in the product-by-process claim is the same as or obvious from a product of the prior art, the claim is unpatentable even though the prior product was made by a different process." *In re Thorpe*, 777 F.2d 695, 698, 227 USPQ 964, 966 (Fed. Cir. 1985) (citations omitted) (Claim was directed to a novolac color developer. The process of making the developer was allowed. The difference between the inventive process and the prior art was the addition of metal oxide and carboxylic acid as separate ingredients instead of adding the more expensive pre-reacted metal carboxylate. The product-by-process claim was rejected because the end product, in both the prior art and the allowed process, ends up containing metal carboxylate. The fact that the metal carboxylate is not directly added, but is instead produced in-situ does not change the end product.).

ONCE A PRODUCT APPEARING TO BE SUBSTANTIALLY IDENTICAL IS

FOUND AND A 35 U.S.C. 102/103 REJECTION MADE, THE BURDEN SHIFTS TO THE APPLICANT TO SHOW AN UNOBVIOUS DIFFERENCE

"The Patent Office bears a lesser burden of proof in making out a case of *prima facie* obviousness for product-by-process claims because of their peculiar nature" than when a product is claimed in the conventional fashion. *In re Fessmann,* 489 F.2d 742, 744, 180 USPQ 324, 326 (CCPA 1974). Once the Examiner provides a rationale tending to show that the claimed product appears to be the same or similar to that of the prior art, although produced by a different process, the burden shifts to applicant to come forward with evidence establishing an unobvious difference between the claimed product and the prior art product. *In re Marosi,* 710 F.2d 798, 802, 218 USPQ 289, 292 (Fed. Cir. 1983) (The claims were directed to a zeolite manufactured by mixing together various inorganic materials in solution and heating the resultant gel to form a crystalline metal silicate essentially free of alkali metal. The prior art described a process of making a zeolite which, after ion exchange to remove alkali metal, appeared to be "essentially free of alkali metal." The court upheld the rejection because the applicant had not come forward with any evidence that the prior art was not "essentially free of alkali metal" and therefore a different and unobvious product.).

Ex parte Gray , 10 USPQ2d 1922 (Bd. Pat. App. & Inter. 1989) (The prior art disclosed human nerve growth factor (b-NGF) isolated from human placental tissue. The claim was directed to b-NGF produced through genetic engineering techniques. The factor produced seemed to be substantially the same whether isolated from tissue or produced through genetic engineering. While the applicant questioned the purity of the prior art factor, no concrete evidence of an unobvious difference was presented. The Board stated that the dispositive issue is whether the claimed factor exhibits any unexpected properties

compared with the factor disclosed by the prior art. The Board further stated that the applicant should have made some comparison between the two factors to establish unexpected properties since the materials appeared to be identical or only slightly different.).

THE USE OF 35 U.S.C. 102/103 REJECTIONS FOR PRODUCT-BY-PROCESS CLAIMS HAS BEEN APPROVED BY THE COURTS

"[T]he lack of physical description in a product-by-process claim makes determination of the patentability of the claim more difficult, since in spite of the fact that the claim may recite only process limitations, it is the patentability of the product claimed and not of the recited process steps which must be established. We are therefore of the opinion that when the prior art discloses a product which reasonably appears to be either identical with or only slightly different than a product claimed in a product-by-process claim, a rejection based alternatively on either section 102 or section 103 of the statute is eminently fair and acceptable. As a practical matter, the Patent Office is not equipped to manufacture products by the myriad of processes put before it and then obtain prior art products and make physical comparisons therewith." *In re Brown*, 459 F.2d 531, 535, 173 USPQ 685, 688 (CCPA 1972).

2114 **Apparatus and Article Claims - Functional Language**

For a discussion of case law which provides guidance in interpreting the functional portion of means-plus-function limitations see MPEP § 2181 - § 2186.

APPARATUS CLAIMS MUST BE STRUCTURALLY DISTINGUISHABLE FROM